

Molecular Physiology of P2X Receptors

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North, R. Alan. Molecular Physiology of P2X Receptors. *Physiol Rev* 82: 1013–1067, 2002; 10.1152/physrev.00015.2002.—P2X receptors are membrane ion channels that open in response to the binding of extracellular ATP. Seven genes in vertebrates encode P2X receptor subunits, which are 40–50% identical in amino acid sequence. Each subunit has two transmembrane domains, separated by an extracellular domain (~280 amino acids). Channels form as multimers of several subunits. Homomeric P2X₁, P2X₂, P2X₃, P2X₄, P2X₅, and P2X₇ channels and heteromeric P2X_{2/3} and P2X_{1/5} channels have been most fully characterized following heterologous expression. Some agonists (e.g., $\alpha\beta$ -methylene ATP) and antagonists [e.g., 2',3'-O-(2,4,6-trinitrophenyl)-ATP] are strongly selective for receptors containing P2X₁ and P2X₃ subunits. All P2X receptors are permeable to small monovalent cations; some have significant calcium or anion permeability. In many cells, activation of homomeric P2X₇ receptors induces a permeability increase to larger organic cations including some fluorescent dyes and also signals to the cytoskeleton; these changes probably involve additional interacting proteins. P2X receptors are abundantly distributed, and functional responses are seen in neurons, glia, epithelia, endothelia, bone, muscle, and hemopoietic tissues. The molecular composition of native receptors is becoming understood, and some cells express more than one type of P2X receptor. On smooth muscles, P2X receptors respond to ATP released from sympathetic motor nerves (e.g., in ejaculation). On sensory nerves, they are involved in the initiation of afferent signals in several viscera (e.g., bladder, intestine) and play a key role in sensing tissue-damaging and inflammatory stimuli. Paracrine roles for ATP signaling through P2X receptors are likely in neurohypophysis, ducted glands, airway epithelia, kidney, bone, and hemopoietic tissues. In the last case, P2X₇ receptor activation stimulates cytokine release by engaging intracellular signaling pathways.

I. INTRODUCTION

ATP is present outside cells. Many cell types release ATP, and the mechanisms and physiological circumstances range from relatively well understood to quite controversial (see Refs. 51, 135, 161, 191, 407, 485). Extracellular ATP acts on cell surface receptors of the P2X and P2Y types (53, 347); it may be involved in phosphorylation reactions through ectokinases (110), and it is rapidly degraded by a series of cell surface enzymes to ADP, AMP, and adenosine (523), the last of which is taken back into cells by a specific transporter (9).

The first cDNAs encoding P2X receptor subunits were isolated in 1994. Their expression in heterologous cells substantiated the view that P2X receptors were ion channels gated by ATP. This review deals first with the molecular properties of the P2X receptors when heterologously expressed and is organized into sections according to the identified subunits. The second part of the review deals with the functional properties of P2X receptors expressed in native cells, reporting studies to establish their molecular identity and physiological role. The emphasis here is on work that most directly addresses the molecular characterization of the receptors; ideally, such studies would use the approaches of 1) gene knock-out, 2) antisense knock-down, 3) biophysical methods such as the kinetics of the responses or the permeation properties of the channel, and 4) quantitative pharmacological studies with a range of agonists and antagonists. Although the era of the molecular physiology of P2X receptors began with the cloning of the cDNAs, there was already a substantial and highly credible body of work that showed the importance of signaling by extracellular nucleotides in many tissue and organ systems. This has been extensively reviewed previously (1, 50, 53, 376).

II. THE P2X RECEPTOR GENE FAMILY

There are seven genes for P2X receptor subunits. Their chromosomal locations are summarized in Table 1. P2X₄ and P2X₇ subunit genes are located close to the tip of the long arm of chromosome 12 (12q24.31), where 230 kb of genomic DNA contain also the gene for calmodulin-dependent kinase type II. On the basis of radiation hybrid mapping, they were judged to be <130 kb apart (46). In fact, the genes are adjacent in the genomes of humans (23,492 bp separating) and mice (26,464 bp separating; chromosome 5). This presumably reflects gene duplication, and P2X₄ and P2X₇ subunits are among the most closely related pairs in amino acid sequences (Figs. 1 and 2). P2X₁ and P2X₅ genes are also very close together (and close to the gene encoding the vanilloid receptor VR1) on the short arm of chromosome 13 (Table 1). The remaining genes are on different chromosomes (Table 1).

TABLE 1. *Chromosomal localization of human P2X receptors*

Subunit	Chromosome	Accession Nos.	Reference Nos.
P2X ₁	17p13.2	X83688	472
P2X ₂		AF190826	292
P2X ₃	11q12	Y07683	147
P2X ₄	12q24.31	Y07684	145
P2X ₅	17p13.3	AF016709	271
P2X ₆	22q11	AB002059	471
P2X ₇	12q24.31	Y09561	380

Accession numbers and references are those for the original submission of cDNA sequences. Chromosomal localizations are from human genome databases (<http://www.sanger.ac.uk> and Ref. 475). P2X₂ chromosomal location is not yet determined. The mouse gene is located on chromosome 5, in a region that is syntenic with the extreme end of the long arm of human chromosome 12 (some 6 MB from the P2X₄ and P2X₇ genes).

The genes vary considerably in size (e.g., mP2X₃: 40 kb, Ref. 434; hP2X₆: 12 kb, Ref. 471). The full-length forms have 11–13 exons, and all share a common structure, with well-conserved intron/exon boundaries (Fig. 1). Many spliced forms of the receptor subunits (or fragments thereof) have been described (Table 2); the majority of these represent simple forms in which one or more exons have been spliced out, although some have altered exons through the use of alternative donor/acceptor sites. Several full-length nonmammalian vertebrate sequences are available (Fig. 2). There are no reports of homologous sequences from invertebrate species, although there is considerable functional evidence that extracellular ATP and other nucleotides can directly gate ion channels in invertebrates including protists (8, 71, 241, 372).

III. THE P2X RECEPTOR PROTEIN FAMILY

A. Amino Acid Sequence

The P2X subunit proteins are 384 (cP2X₄) to 595 (P2X₇) amino acids long. Each has two hydrophobic regions of sufficient length to cross the plasma membrane (37, 346, 472) (Fig. 1); the first of these extends from residue 30 to 50, and the second from residue 330 to 353 (numbers refer to the rat P2X₂ receptor). These hydrophobic regions are separated by the bulk of the polypeptide; considerable evidence presented below indicates that much, perhaps all, of this lies on the extracellular aspect of the membrane. The NH₂ and COOH termini are therefore presumed to be cytoplasmic. The COOH-terminal regions diverge in sequence considerably. Considering the region of the protein which includes the two transmembrane domains and the intervening extracellular domain (i.e., amino acids 30–353 of P2X₂), the proteins are from 40 to 55% pairwise identical (Table 3). The P2X₄ sequence is most closely related to more of the other

		Exon 2	
P2X1	MARRLQDELSA-FFFEYDTPRMVLVRNKKVGVIFRLIQLVVLVYVIGWVFVYERQYQTSS		59
P2X2	MVRRLARGCWS-AFWDYETPKVIVVRNRRIGFVHRMVQLLLILYFVWYVFIVOKSYQDSE		59
P2X3	-----MNCIS-DFFTYETTKSVVKSWTIGIINRAVOLLIISYFVGWVFLHEKAYQVRD		53
P2X4	MAGCCSVLG-S-FLFEYDTPRIVLIRSRRVGLMNRVOLLILAYVIGWVFVWEKAYQETD		58
P2X5	MGQAANKGFV-LSLFDYKTAKFVVAKSKKVGLLYRVLLIILYLLIWWFLIKKSYQDID		59
P2X6	MASAVAAALVSWGFLDYKTEKYVMTRNCWVGISORLLQLGVVVYVIGWALLAKKGYQEDW		60
P2X7	MPACCSWN-----DVFOYETNKVTRIQSVNYGTIKWILHMTVFSYVS-FALMSDKLYQRKE		55
		Exon 3	
P2X1	D-LISSVSVKIKGLAVT---QLQGLG-PQ----VWDVADYVFPAGHDSSFFVMTNFIVTPQ		111
P2X2	TGPESIITKVKGITMS-----EDK-----VWDVEEYVKPEGGSVSIIITRIEVTPS		107
P2X3	TAIESSVVTIKVKGFGRY-----ANR-----VMDVSDYVTEPQGTSTVFVITIKMIVTEN		101
P2X4	S-VVSSVTTKAKGVAVT--NTSQLG-FR----IWDVADYVIPAQEENSFLFIMTNMIVTVN		110
P2X5	TSLSASAVVTKVKGVAYTNTTMLGER-----LWDVADFVIPSQGENVFVVTNLTIVTPN		112
P2X6	MDPQISVITIKLKGVSVTQVKELEKR-----LWDVADFVRPSQGENVFVLTNLTIVTPA		113
P2X7	P-LISSVHTIKVKGVAEVTENVTEGGVTKLVHGIFDTADYTLFLOG-NSFFVMTNLYLKSEG		113
		Exon 4	Exon 5
P2X1	QTQGHCAENPE-GG-ICDDSGCTPKAERKAQGI RTGNCVP-FNGTVK-TCEIFGWCPV		167
P2X2	OTLGTGPESMRVHSSTCHSDDDCIAGQOLDMOGNGIRTGHCVPYYHGDSK-TCEVSAWCPV		166
P2X3	QMCGFCPENEEKYR--CVSDSQ--GPERFPGGGIITGRGVN-YSSVLR-TCEIQGWCPV		155
P2X4	QTQSTCEIPDKTS-IGNSDADCTPGSVDTSSGVATGRGVP-FNESVK-TCEVAWCPV		167
P2X5	QRQGICAEEREGIPDGECSEDDCHAGESVVAHGHLKTGRCLR-VGNSTRGTCEIFAWCPV		171
P2X6	QVQGRGPEHPSVPLANCAWADEDCPEGEMGTYSHGIGTQCVAFNGTHR-TCEIWSWCPV		171
P2X7	QEQKLCPEYPSRGK-QCHSDQGCIGWMDPQSKGIOTGRCIP-YDQKRK-TCEIFAWCPA		170
		Exon 6	Exon 7
P2X1	EVDDKIPSPALLREANFTLFIKNSISFPRFKVNRNVLVEEVNGTYMKKCLYHKIOHPLC		227
P2X2	EDG-TSDNHFLGKMAPNFTILIKNSIHYPKFKFSKGNIASQKSD-YLKHCTFDQDSDPYC		224
P2X3	EVD-TVEMPIM-MEAENFTIFIKNSIRFPLNFEEKGNLLPNLTDKDIKRCRPHPEKAPFC		209
P2X4	ENDVGVPPTAFLKAAENFTLLVKNNIWPKFNFSKRNILPNITTSYLKSCIYNAQTDPPC		227
P2X5	ETK-SMPTDPLLKDAESFTISIKNFIKFNFSKANVLETDNKHFLKTCFHSSTN-LYC		229
P2X6	ESS-AVPRKPLLAQAKNFTLFIKNTVTFNKFNSRTNALDNTWNTYPKYCLYDSLSPYC		230
P2X7	BEGKEAPRPALLRSANFTVLIKNIDFPGHNYTTRNIPGMNIS----CTPHKTWNPOC		226
		Exon 8	
P2X1	FVFNLYGVVRESGODFRSLAEKGGVVGITIDWKCDLDWHVRHCKPIYQFHGI---YGERN		284
P2X2	PIFRILGFIVEKAGENFTELAHKGGVIGVIINWNCDDLSESECNPKYSFRRILD--PKYDP		282
P2X3	PIIRVGDVVKFAGQDFAKLARTGGVLGKIGWVCDLDKAWDQCIPIKYSFRRILDGVSEKSS		269
P2X4	PIFRILGTIVGDAGHSFOEMAVEGGIMGIQIKWDCNLDRAASLCLPRYSFRRILDRDLEHN		287
P2X5	PIFRILGSIVRWAGADFQDIALKGGVIGIYIEWDCDLKAASKCNPHYFENRDLNKHHS-		288
P2X6	FVFRIGDLVAMTGGDFEDLALIGGAVGINIHWDCNLDTKGSDCSPQYSF-QLQE-----		283
P2X7	PIFRILGDIQFQIEGENETEVAVQGGIMGIETIYWDNLDLSWSHRGQPKYSFRRILDDKYTNES		286
		Exon 9	Exon 10
P2X1	LSPGFNFRFARHFVQ-NGTNRRHDFKVFGIHFDILVDGKAGKFDIIPMTTIGSGIGIFG		343
P2X2	ASSGYNFRFAKYYKINGTTTTITLTKAYGIRIDVIVHGQAGKFSLIPTIINLATALTSIG		342
P2X3	VSPGFNFRFAKYYKMENGSEYRTLLKAFGIRFDVLVYGNAGKFNIIPTIISVSAFTSVG		329
P2X4	VSPGFNFRFAKYYRDLAGKEQRTLLTKAYGIRFDIIVFGKAGKFDIIPMTINVGSGLALLG		347
P2X5	ISSGYNFRFARYYRDPNGVEFRDLMKAYGIRFDVIVNGKAGKFSIIPTVINIGSGLALMG		348
P2X6	--RGYNFRFANYWAAASGVESRSLKLYGIRFDILVTGQAGKFIIPTAITVGTGAAWLG		341
P2X7	LFPGYNFRFAKYYKE-NGMEKRTLLKAFGVRFDIIVFGTGKFDITQLVVYIGSTLSYFG		345
		Exon 11	
P2X1	VATVLCIDLLLLH-----ILPKRHYYKQKKFYAEDMGPGEGEDHPV		384
P2X2	VGSFLCDWILLT-----FMNKNKLYSHKKFDKVRTPKHPSSRWPTVL		384
P2X3	VGTVLCIDILLN-----FLKGADHYKARKFEEVTETTLKGTASTNPV		371
P2X4	VATVLCIDVIVLY-----CMKKKYVYRDKKYKYVEDYEQGLSGEMNQ		389
P2X5	AGAFFCDLVLIY-----LIRKSEFYRDKKFEKVRGQKEDANVEVEAN		390
P2X6	MVTFLCDLLLY-----VDREAGFYWRKYEEARAPKATNSA	379	
P2X7	LATVCIDLIINTYASTCCRSRVPSCKCCEPCAVNEYVYRKKEPIVEPKPTLKYSVFD		405
P2X1	ATSSTLGLQENMRTS 399		
P2X2	ALVLGQIPPPPSHYSQDQPPSPSPSGEGPTLGEAELPLAVQSPPRCSISALTEQVVDTLG		444
P2X3	FASDQATVEKQSTDGAYSIGH 393		
P2X5	EMEQRPEDEPLERVQDEQSQELAQSGRKQNSNCQVLLPARFGLRENAIVNVKQSQIL		450
P2X7	EPHIWMVDQQLLGKSLQDVKGQEVPRPQTDFLELSRLSLSLHHSPPPIPGQPEEMQLLQIE		465
P2X2	QHMQRPPVPEPSQDSTSTDPKGLAQL 472		
P2X5	HPVKT 455		
P2X7	AVPRSRDSDPWCQCGNCLPSQLPENRRALEELCCRRKPGQCITTSSELFISKIVLSREALQL		525
P2X7	LLLYQEPLLALEGEAINSKLRHCAYRSYATWRFVSQDMADFAILPSCCRWKIRKEFPKTQ		585
P2X7	GOYSGFKYPY 595		

FIG. 1. P2X receptor subunits. Alignment of rat amino acid sequences is shown. Open boxes indicate conserved amino acids. Shading indicates conserved cysteines. Solid overlines indicate hydrophobic, membrane-spanning regions. Positions corresponding to the beginning of each exon are indicated. Sequences and gene structure are deduced from NCBI accession numbers P47824 (rP2X₁), 2020424A (rP2X₂), CAA62594 (rP2X₃), CAA61037 (rP2X₄), CAA63052 (rP2X₅), CAA63053 (rP2X₆), and CAA65131 (rP2X₇).

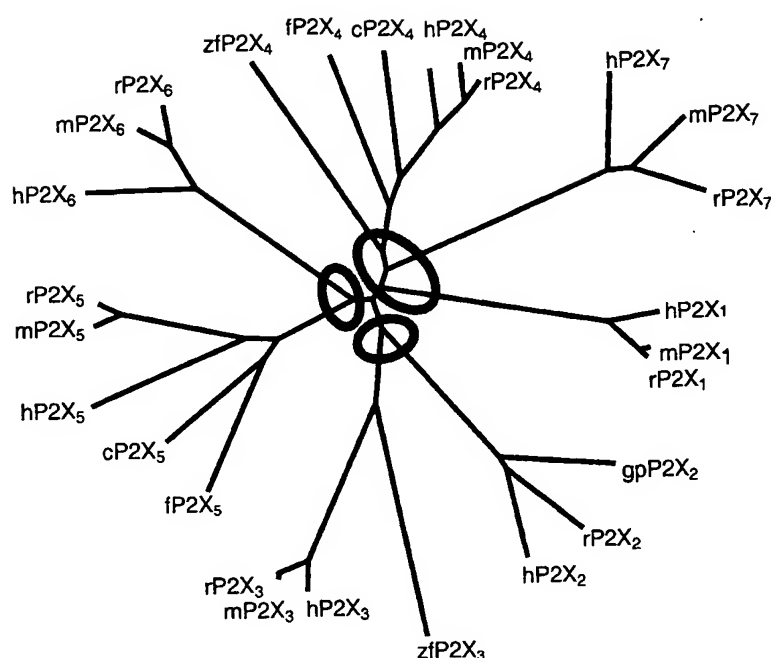


FIG. 2. Dendrogram to show relatedness of 29 P2X receptor subunits. Full-length amino acid sequences were aligned with Clustal W using default parameters. The dendrogram was constructed with TreeView. h, Human (*Homo sapiens*); r, rat (*Rattus norvegicus*); m, mouse (*Mus musculus*); gp, guinea pig (*Cavia porcellus*); c, chicken (*Gallus gallus*); zf, zebrafish (*Danio rerio*); bf, bullfrog (*Rana catesbeiana*); x, claw-toed frog (*Xenopus laevis*); f, fugu (*Takifugu rubripes*). The ellipses indicate the apparent clustering by relatedness into subfamilies.

forms, and the P2X₇ sequence is least like the others; these observations are true whichever species are considered (Table 3).

The amino acid identity between P2X receptor subunits is distributed throughout the extracellular domain, a striking feature of which is the conservation of 10 cysteine residues among all known receptors (Fig. 1). These are not obviously conserved in blocks with respect to exonic structure; the first half of the domain contains six cysteines (exons 2, 4, and 5), and the four further cysteines are in sequence encoded by exons 7 and 8. It is generally thought that such cysteines in an extracellular location would be oxidized and thus contribute to the tertiary structure of the protein by disulfide bond formation; there is no direct evidence for this in the sense that treatment with reducing agents has no effect on channel function (74, 114, 379). The possible pattern of disulfide bond formation has been approached by systematic cysteine to alanine substitutions. Clyne et al. (74) compared the effects of such substitutions (in the rat P2X₂ receptor) on sensitivity to ATP and potentiation by zinc and found that the results could be grouped according to residue. Ennion and Evans (114) carried out similar experiments for the human P2X₁ receptor, but used a clever additional approach. This was to demonstrate that the receptor became accessible to labeling by MTSEA-biotin after a cysteine to alanine mutation, presumably as a result of a free sulfhydryl becoming available. By adding a second cysteine to alanine mutations, they were able in some cases to assign partners, although not all possibilities were tested. The results of these experiments are illustrated schematically in Figure 3. The finding by Ennion and

Evans (114) that Cys-124, Cys-130, Cys-147, and Cys-158 (rat P2X₂ numbering) were able to interact promiscuously might indicate that these residues are clustered, as would be expected for a metal ion binding site. However, the ion seems not to be zinc (see Ref. 74).

There is no reported homology of sequence between P2X receptors and other proteins, although a similarity has been suggested to class II aminoacyl-tRNA synthetases (138). This similarity is mostly between the predicted secondary structure of the second half of the extracellular domain (residues 170–330) and that known from X-ray crystallography of the synthetases, which form their catalytic site from a seven-stranded antiparallel β -pleated sheet (92). It was stated that the first half of the extracellular domain (residues 110–170) may provide a metal ion binding site (138), but there is no evidence that the cysteines are involved in this (74).

B. Glycosylation and Membrane Topology

All the P2X receptor subunits have consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr), and some glycosylation is essential for trafficking to the cell surface. The P2X₁ subunit sequence has five such consensus sites, four of which are conserved among human, rat, and mouse sequences (asparagines 153, 184, 284, 300 in rat P2X₁). These four sites can all be glycosylated (341). The P2X₂ subunit has three such sites (asparagines 182, 239, and 298 in rat P2X₂), and all are glycosylated in oocytes (340) and HEK293 cells (459). The consequences of removal (by tunicamycin) or prevention (by mutagen-

TABLE 2. *Splice variants of P2X receptors*

Subunit	Name	Description	Accession Nos.	Reference Nos.
P2X ₁				
Human		Exon 10 missing		179
		Exon 6 missing first 17 aa		156
Rat		Exon 6 missing	AAF73925	349a
	RP-2	Exons 1–6 missing (also 39 5'-bases added)	S50860	353
P2X ₂				
Human ^b	P2X2B	Exon 12 missing 201 bp	AAD42948	292
	P2X2C	Exon 3 missing	NP_057402	292
	P2X2D	Insertion between exons 10 and 11	AAF19173	292
	P2X2H	Misses last 22 aa of exon 1, exon 2, and exon 3	AAF74203	
	P2X2I	Exons 2, 3, and 4 replaced by 42 aa	AAF74204	
Rat	P2X2b	Exon 12 missing 207 bp	CAA71499	418
	P2X2c	Exon 2 missing first 7 aa	CAA71500	418
	P2X2d	Exon 2 missing first 12 aa	CAA71501	418
	P2X2e	Fragment, missing exon 12	AAC72285	
	P2X2f	Fragment, alternative exon 12	AAC72286	
	P2X2g	Fragment, alternative exon 12	AAC72287	
	P2X2-3	Exon 6 missing	AAB94570	
Guinea pig	P2X2-1	Exon 9 missing	AAC08992	361
	P2X2-2	Exons 9 and 10 missing	AAC08993	361
	P2X2-3	27 aa inserted between exons 8 and 9	AAC08994	361
P2X ₃				
Rat	P2X3b	Exon 3 missing	AAD47381	
P2X ₄				
Mouse	P2X4a ^a	Exon 6 missing	AAC95602	464
	P2X4b ^a	Exon 6 missing	CAB90750	464
	P2X4c	Exon 10 missing	CAB90751	464
	P2X4d	Exon 6 and exon 10 missing	CAB90752	464
Human		Exons 1 and 2 replaced by in-frame Hsp-90 homolog	JC6543	96
P2X ₅				
Human	P2X5a	Exon 10 missing	AAB08576	
	P2X5b	Exons 3 and 10 missing	AAB08577	
P2X ₆				
Human	P2XMN1	Exon 1 missing 26 aa		471
	P2XM-AL1	Exon 1 missing 78 bp from 3'-end		471
	P2XM-AL2	Exon 10 missing		471
	P2XM-AL3	Exons 10 and 11 missing		471

^a These are identical. The designation P2X4a was applied by Simon et al. (418) to the full-length mP2X4 (CAB90749). ^b "Full-length" hP2X2 was called hP2X2A by Lynch et al. (292). Accession Nos. refer to NCBI protein database accession numbers. References are cited where published; otherwise, there was direct deposit to Genbank. Spliced forms of the P2X₇ receptor have not been reported. aa, Amino acids.

TABLE 3. *Pairwise identity of P2X receptor subunits (considering the amino acid sequence of transmembrane regions and large extracellular loop)*

	P2X ₁	P2X ₂	P2X ₃	P2X ₄	P2X ₅	P2X ₆	P2X ₇
P2X ₁	100	40.6	47.9	50.3	44.7	46.2	45.1
	100	40.5	46.4	50.6	45.5	46.8	44.9
P2X ₂		100	51.1	50.5	46.9	42.7	41.0
		100	51.1	50.5	46.9	42.7	41.0
P2X ₃			100	48.6	49.3	43.2	44.7
			100	49.2	47.0	41.4	43.1
P2X ₄				100	55.4	47.6	48.6
				100	53.5	47.3	49.8
P2X ₅					100	48.5	42.0
					100	49.2	42.0
P2X ₆						100	41.0
						100	39.2
P2X ₇							100

In each cell, the upper number pertains to the human and the lower number to the rat sequences.

esis) of glycosylation have been studied. Receptors in which any two of the three sites are glycosylated appear at the cell surface and are fully functional. Receptors in which only one site is glycosylated give barely detectable currents in response to ATP, and channels with no sites glycosylated give no current. These double and triple mutant receptors are retained within the cell, as detected by immunohistochemistry of a COOH-terminal epitope tag (340), or immunoprecipitation of cell surface membrane protein labeled with sulfo-NHS-LC-biotin labeling (459). The other P2X receptor subunits also have consensus sequences for N-linked glycosylation; these are well conserved in their positions among species variants but incompletely conserved among the receptors (P2X₃, four sites; P2X₄, six sites; P2X₅, two sites; P2X₆, three sites; P2X₇, three sites).

The membrane topology of the protein has also been addressed by determining the location of glycosylation sites; thus the studies on the P2X₂ receptor indicate that

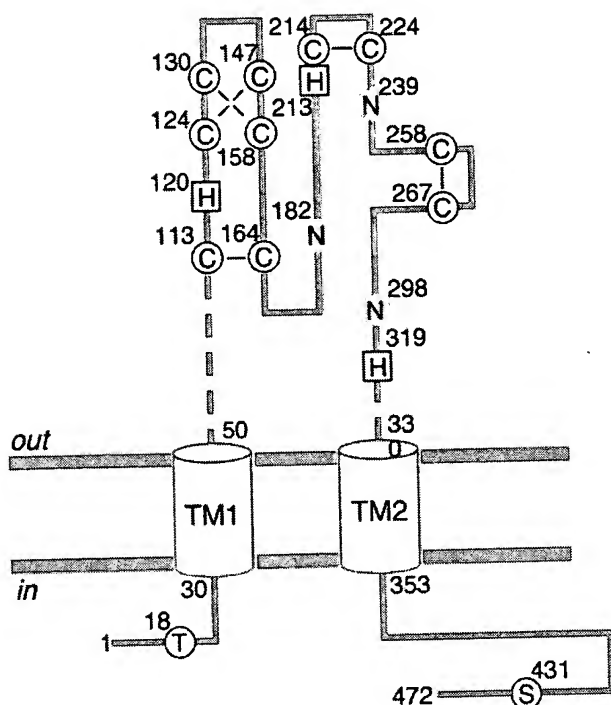


FIG. 3. Glycosylation, phosphorylation, and possible disulfide bonding of P2X₂ receptors. Solid circles (N) indicate the three sites that are glycosylated in the native P2X₂ receptor (data from Refs. 340, 459, 460). Open circles (T, S) indicate the positions of Thr-18 (threonine phosphorylated by protein kinase C; Ref. 33) and Ser-431 (serine phosphorylated by protein kinase A; Ref. 66). Open circles (C) indicate the 10 conserved cysteines. Alanine substitution and MTSEA-biotin labeling experiments indicate possible disulfide bond formation; data are from P2X₁ receptor (114) and from P2X₂ receptor (74). Open squares (H) indicate histidine residues involved in zinc (His-120, His-213) and proton (His-319) binding (data from Ref. 74).

asparagines 182, 239, and 298 are all localized to the extracellular domain (Fig. 3). Site-directed mutagenesis has been used to introduce new consensus sites into a background P2X₂ receptor in which the three natural sites have been removed (340, 459). These studies provide direct support for the proposed topology, with a large extracellular domain between the two membrane-spanning regions. Further evidence that the NH₂ and COOH termini reside on the same side of the membrane comes from studies in which two cDNAs have been joined in tandem (340, 442, 460). Such constructs express fully functional channels, and point mutations in one or other of the concatenated domains indicate that both contribute to the channel (340, 442). Finally, confocal immunofluorescence microscopy has been carried out on HEK293 cells transfected with P2X₂ receptors carrying a FLAG epitope at the NH₂ or COOH terminus; in either case, the epitope was accessible to antibody only when the cells had been permeabilized (460).

The P2X₇ subunit has a much longer COOH terminus than the other subunits, and this contains an additional

hydrophobic domain (residues 510–530) that is sufficiently long to cross the plasma membrane. There is no published definitive evidence that places the COOH terminus of this receptor inside or outside the cell, but membrane topology algorithms suggest an intracellular location.

C. Multimerization

Evidence for heteromultimeric receptors has come from functional expression studies, whereas although these show that at least two different subunits can contribute to the ion channel, they are inconclusive with regard to the actual number of subunits. Three kinds of biochemical approaches have also been used. Schmalzing and colleagues (341) cross-linked P2X₁ and P2X₃ receptors, either in intact oocytes or after solubilization with digitonin. The receptors were NH₂-terminally tagged with hexahistidine sequences and cross-linked either with 3,3'-dithiobis(sulfosuccinimidyl-propionate) or with bifunctional analogs of the antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS). One of these analogs (CLII) has a flexible spacer between the phenyl group so as to provide up to 3.4 nm between the two pyridoxal aldehyde moieties; it was able to cross-link digitonin-solubilized, purified P2X₁ (or P2X₃) subunits almost quantitatively to homotrimers, and this was reversed to monomers by dithionite, which cleaves the azo bonds of CLII. Cross-linking with CLII of octylglucoside-solubilized P2X₁ receptors led to the appearance of hexamers and trimers, but not intermediate forms (341).

In a second approach, blue native polyacrylamide gel electrophoresis was used to estimate the molecular mass of the P2X₁ receptor isolated under nondenaturing conditions from digitonin extracts of oocytes. These were almost exclusively trimers, whereas parallel experiments on the muscle type nicotinic receptor (coexpression of α , β , γ , and δ subunits) clearly resolved the expected pentameric structure. Generally consistent results have been reported for rat P2X₇ receptors (239).

The third approach used the hexahistidine-tagged ectodomain of the rat P2X₂ receptor (residues Lys-53 to Lys-308). This was expressed in *Escherichia coli*, solubilized in urea, and purified by nickel-affinity chromatography (240). After sulfitolysis and refolding, the protein was photoaffinity labeled with [α -³²P]ATP; the labeling was prevented by an excess of cold ATP and by suramin (1 μ M) and cibacron blue (10 μ M). The molecular size of the labeled protein was estimated by equilibrium sedimentation centrifugation as 132 kDa, which is about four times the calculated size of the ectodomain (29 kDa). Obviously, one difficulty of this approach is knowing whether the ectodomain is correctly refolded and whether the ectodomain alone can reconstitute the original ATP binding site. In fact, more recent

work by Egan, Voigt, and colleagues (461) indicates that residues critical for multimerization are in or near the second membrane-spanning segment (461), which was not present in the ectodomain experiments.

Voigt, Egan, and colleagues (460, 462) have also determined which pairs of subunits are potentially able to coassemble. The approach was based on coimmunoprecipitation of epitope-tagged subunits after expression in HEK293 cells (460, 462). Table 4 summarizes their results, which are also consistent with the findings of others with respect to the P2X₂/P2X₃ (374), P2X₄/P2X₆ (269), and P2X₁/P2X₅ (270, 447, 463). Thus at one extreme P2X₇ subunits will coassemble with no others (in this biochemical test); they are also the most distinct in sequence (Table 4). P2X₅ receptors will assemble with any others, except P2X₇.

In summary, the biochemical evidence that the protein readily forms stable trimers and hexamers is suggestive that the intact receptor assembles from three or six subunits in heterologous expression systems. However, there are two types of caveat. First, similar approaches resulted in similar conclusions for the large-conductance mechanosensitive channel (mscL) of *E. coli* (27); this is a channel in which the subunits have a similar transmembrane topology to that proposed for P2X subunits. Electron microscopic images of two-dimensional crystals of reconstituted mscL channels were also interpreted as hexamers (396), but subsequent crystallization of the *Mycobacterium tuberculosis* mscL shows that this channel actually forms as a pentamer (59). Second, assembly in native cells may be influenced significantly by associated proteins that are not present in heterologous expression systems.

IV. HETEROLOGOUS EXPRESSION OF CLONED RECEPTORS

A. Homomeric P2X₁ Receptors

A cDNA encoding the P2X₁ receptor was isolated by direct expression in *Xenopus* oocytes, beginning with a

cDNA library made from rat vas deferens (472). The deduced protein has 399 amino acids. It was noted by Valera et al. (472) that the database already contained a cDNA (RP-2) identical in sequence to part of the P2X₁ receptor cDNA (Table 2); RP-2 cDNA was isolated by subtractive hybridization from thymocytes undergoing apoptosis (353). Human and mouse cDNAs have also been cloned and expressed (473).

1. Agonists

ATP-gated channels express well in oocytes and HEK293 cells after injection or transfection with the P2X₁ subunit cDNA (121, 472, 495). Approximately equal currents can be elicited by ATP or $\alpha\beta$ -methylene ATP ($\alpha\beta$ meATP), each having an EC₅₀ close to 1 μ M (121, 472). 2',3'-O-(benzoyl-4-benzoyl)-ATP (BzATP) is also an effective agonist (25, 121); it is particularly potent when calcium flux is measured, with an EC₅₀ in the low nanomolar range (25). The human receptor was cloned from urinary bladder and is basically similar in properties to the rat receptor; both resemble closely the responses of smooth muscle cells of the vas deferens or bladder (122, 473). The most striking property of the P2X₁ receptor is the mimicry of the agonist actions of ATP by $\alpha\beta$ meATP, which distinguishes P2X₁ and P2X₃ receptors from the other homomeric forms. $\beta\gamma$ MeATP is also useful in this respect; although it does cause maximal currents as large as those evoked by ATP, it activates P2X₁ receptors at concentrations (10 μ M) that are ~30-fold less than those needed to activate homomeric P2X₃ receptors (25, 121, 147, 472).

Ennion et al. (116) have mutated the positively charged residues in the human P2X₁ receptor, in an effort to determine which might contribute to the ATP binding site. They found that the lysines most sensitive to substitution by alanine or arginine were Lys-68 and Lys-70 (corresponding to Lys-69 and Lys-71 in the rat P2X₂ sequence); other positively charged residues closer to the COOH-terminal end of the extracellular loop may also be involved (particularly Lys-309) (116). Negatively charged residues have also been mutated to alanine (117). However, even though these (Asp-86, Asp-89, Glu-119, Asp-129, Glu-160, Glu-168, Asp-170, Glu-183, Asp-262, Asp-264, Asp-316 P2X₁ numbering, see Fig. 1) are highly conserved among all P2X receptors, in no case did the substitution by alanine cause any significant change in the sensitivity to ATP.

The deletion of one leucine residue at the inner end of the second transmembrane domain results in a receptor that does not express and a dominant negative phenotype when the mutated form is coexpressed with wild-type P2X₁ receptors (352); this mutation was made because it was detected in a 6 yr old with a bleeding diathesis that appeared to be due to deficient platelet aggregation, but cause and effect remain obscure. P2X₁

TABLE 4. Potential coassembly of P2X receptor subunits

	P2X ₁	P2X ₂	P2X ₃	P2X ₄	P2X ₅	P2X ₆	P2X ₇
P2X ₁	+	+	+	—	+	+	—
P2X ₂		+	+	—	+	+	—
P2X ₃			+	—	+	—	—
P2X ₄				+	+	+	—
P2X ₅					+	+	—
P2X ₆						—	—
P2X ₇							+

P2X receptor subunits carrying either one of two epitope tag units were expressed in pairs of HEK293 cells. +, Subunits immunoprecipitated with antibody to one epitope could be detected with an antibody to the second epitope. [Data from Torres et al. (462).]

receptors are expressed by platelets (see sect. v13). Finally, a spliced form of the hP2X₁ receptor that lacks most of exon 6 (including the conserved glycosylation site Asn-184) has been found in platelets and megakaryocyte cell line (156). When expressed in fibroblasts and studied by calcium imaging, this receptor showed a much reduced sensitivity to $\alpha\beta$ meATP.

2. Antagonists/blockers

P2X₁ receptors are blocked by suramin and PPADS (121), but there are now newer antagonists that are more P2X₁ selective. MRS2220 (cyclic pyridoxine- α 4,5-monophosphate-6-azo-phenyl-2',5'-disulfonate) blocks at ~ 10 μ M but has no effect on currents evoked at P2X₂ or P2X₄ receptors (or human P2Y₂, human P2Y₄, or rat P2Y₆) (210). The structures of the main antagonists are shown in Figure 4. Certain suramin analogs also exhibit a relatively high affinity for P2X₁ receptors: 8,8'-carbonylbis(imino-3,1-phenylene carbonylimino)bis(1,3,5-naphthalenetrisulfonic acid) (NF023) blocks P2X₁ receptors more effectively than P2X₂, P2X₃, and P2X₄ receptors (432), and 8,8'-carbonylbis(imino-4,1-phenylene carbonylimino)bis(1,3,5-naphthalenetrisulfonic acid) (NF279) blocks P2X₁ receptors in oocytes with an IC₅₀ of 50 nM (249). The PPADS analog pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulfonate) (PPNDS) blocks P2X₁ receptors with an IC₅₀ of ~ 10 nM (266). Another useful antagonist at P2X₁ receptors is 2',3'-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP), which has an IC₅₀ of ~ 1 nM (483). Among the other receptors, only the P2X₃ homomers and P2X₂/P2X₃ heteromers are similarly sensitive. This action of TNP-ATP is shared by TNP-GTP, TNP-ADP, and TNP-AMP, but not by TNP-adenosine. Finally, di-inosine pentaphosphate (Ip5I) has been described as a selective antagonist at recombinant P2X₁ receptors (242).

Little information is available with respect to the regions of the receptor involved in antagonist binding. Ennion et al. (116) have determined the effects on suramin antagonism of mutating positively charged amino acids in the extracellular loop. In oocytes expressing human P2X₁ receptors, the block by suramin was slightly increased in receptors with K70R, K215R, and K309R substitutions and decreased in the case of R202A and R292A.

3. Permeation properties

The homomeric P2X₁ receptor is a cation-selective channel that shows little selectivity for sodium over potassium (122). It has a low permeability to larger organic cations such as Tris ($P_{\text{Tris}}/P_{\text{Na}}$ 0.18) or *N*-methyl-D-glucamine ($P_{\text{NMDG}}/P_{\text{Na}}$ 0.04), at least when tested with brief agonist applications (see below). It has a relatively high permeability to calcium, as estimated from reversal potentials in bi-ionic conditions ($P_{\text{Ca}}/P_{\text{Na}}$ 4 in 112 mM Ca,

corrected for ionic activities) (122). Extracellular calcium has little or no inhibitory effect on P2X₁ receptor currents, and this is in marked contrast to the P2X₂ receptor (122). Extracellular acidification inhibits currents at P2X₁ receptors. There are only preliminary reports of the single-channel currents at P2X₁ receptors; the unitary conductance was ~ 18 pS (122, 472).

4. Desensitization/inactivation

Desensitization means the decline in the current elicited by ATP during the continued presence of ATP. The time domain is important; in some P2X receptors this decline occurs in milliseconds (fast desensitization: P2X₁, P2X₃), and in others it occurs 100–1,000 times more slowly (slow desensitization: P2X₂, P2X₄). Figure 5 summarizes the fast and slow desensitization observed for the six P2X receptors that express as homomers in HEK293 cells.

P2X₁ receptors undergo fast desensitization when the agonist application is continued for more than several hundred milliseconds (Fig. 5). The desensitization is not marked at lower concentrations (less than or equal to EC₅₀) but becomes prominent at concentrations above 1 μ M. Recovery from desensitization is extremely slow; second and subsequent applications of ATP do not elicit as large currents as the first application, and such subsequent applications must be made at long intervals (> 15 min) for reproducible responses to be obtained.

The consequences of desensitization can be probed with respect to the detection of functional effects of ATP. The human leukemia cells (HL60) and rat basophilic leukemia cells (RBL) express P2X₁ receptor mRNA and protein, but inward currents in response to extracellular ATP can only be observed after treating the cells with apyrase (45). This surprising observation suggested that ATP was being continuously released from the cells (which was also shown directly by the luciferin-luciferase assay), and responses to exogenous ATP were not observed because the receptor was desensitized. Treatment with apyrase allowed the receptors to recover from desensitization. In view of the increasing number of cell types shown to release ATP (see Refs. 135, 178, 407, 485), this is likely to be a considerable experimental problem in a wide range of tissues.

The marked contrast in the kinetics of desensitization between P2X₁ and P2X₂ receptors prompted a series of experiments with chimeric constructs in an effort to map the domains involved (495). These experiments indicated that desensitization required two regions of the P2X₁ receptor; if either region was replaced by the equivalent segment from the P2X₂ receptor, then desensitization no longer occurred. Each region is 34 amino acids long, comprising the transmembrane segment and the contiguous residues (~ 14) on its intracellular aspect.

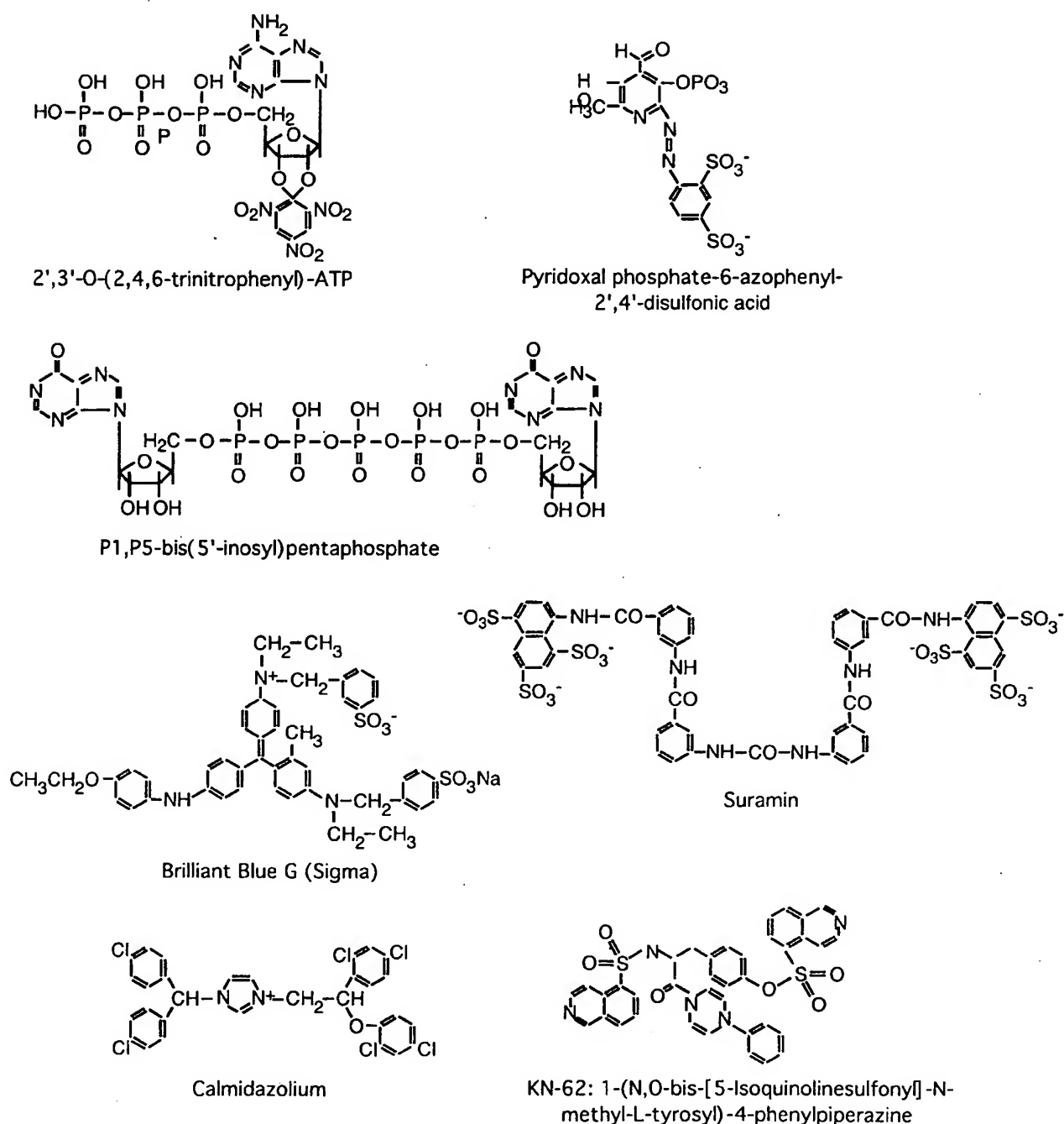


FIG. 4. Structural formulas for several antagonists used in the study of P2X receptors.

These results suggest that closure of the channel during the continued presence of the agonist requires concerted conformational changes involving both transmembrane segments.

Mutations of positively charged residues in the extracellular loop of the human P2X₁ receptor can also have dramatic effects on desensitization. The substitution K68A produces a receptor in which desensitization is greatly slowed (~100-fold), and smaller effects were seen

for R292K, K309A, and K309R. Activation of P2X receptors with these mutations also requires much higher concentrations of ATP (see above). Parker (359) found that the rate of desensitization of wild-type P2X₁ receptors stably expressed in HEK293 cells slowed from ~60 ms to several seconds when the cells were passaged in culture; this change was not seen in M332I and T333S mutations, and it was reversed by cytochalasins B and D (5 μ M, 2–4 h). The threonine residue at position 18 of the P2X₁

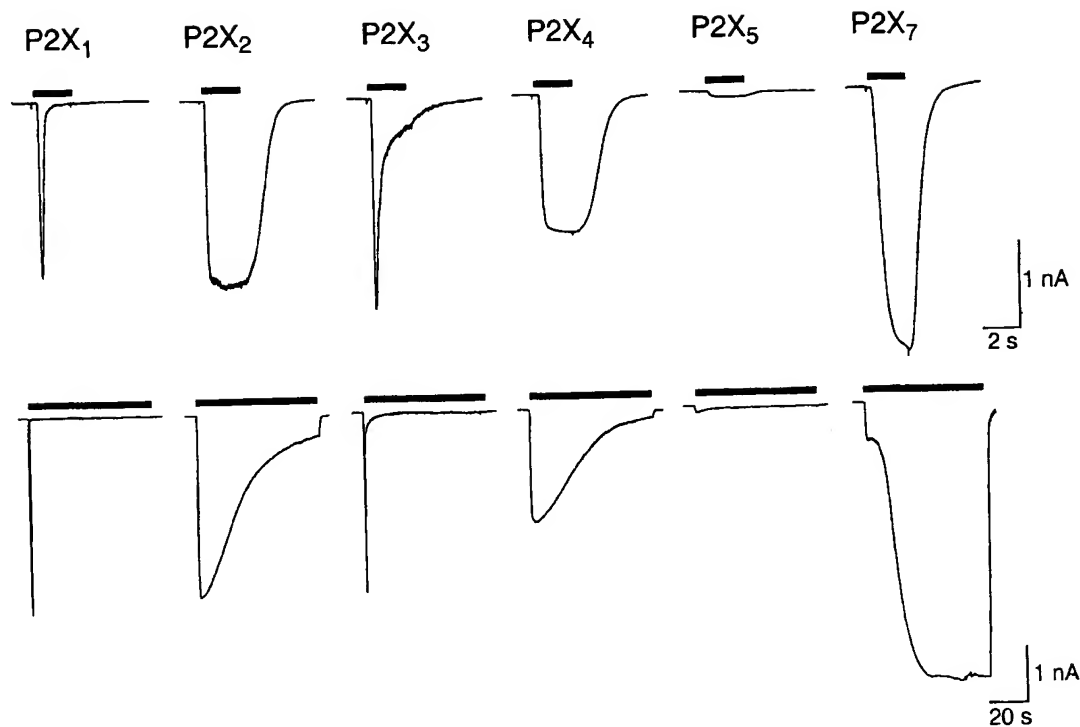


FIG. 5. Fast (*top*) and slow (*bottom*) desensitization compared for homomeric rat P2X receptors. Note 10-fold difference in time scale. Fast desensitization is observed only with P2X₁ and P2X₃; brief applications (2-s duration) of ATP (30 μ M, except 1 mM for P2X₇). Slow desensitization is observed for P2X₂ and P2X₄; more prolonged applications (60-s duration) of ATP (30 μ M, except 1 mM for P2X₇). HEK293 cells were transfected with 1 μ g/ml cDNA (each in pcDNA3.1) 48 h before these whole cell recordings were made. In all cases except P2X₇, the response shown is that seen for the first application of ATP to that cell. For P2X₇, one 2-min application of ATP had been made before the application shown. (Figure kindly provided by A. Surprenant.)

receptor is completely conserved and lies in a protein kinase C consensus sequence. Ennion and Evans (115) showed that replacing this threonine by alanine resulted in a receptor that desensitized 10 times faster than the wild-type receptor, but there is no direct evidence that this change results from its inability to be phosphorylated. The residue lies within the domains identified by Werner et al. (495) as being responsible for the swap in desensitization among P2X₁, P2X₂, and P2X₃ receptors.

Adenoviral expression of a P2X₁ receptor-green fluorescent protein (GFP) construct in *vas deferens* shows the receptor to be localized in clusters, with larger ones apposing nerve varicosities (105). Heterologous expression in rat dissociated superior cervical ganglia presented a similar picture (284); these cells normally exhibit a nondesensitizing response to 1-s applications of ATP, so the time course of the appearance of the P2X₁ subunit was followed functionally by the presence of a desensitizing current in response to $\alpha\beta$ meATP. Exposure to $\alpha\beta$ meATP for \sim 60 s resulted in a loss of GFP from the plasma membrane, with its appearance in acidic endosomes (as judged by monensin sensitivity). Ennion and Evans (113) have made similar conclusions; they found

that a 30-min treatment with $\alpha\beta$ meATP (100 μ M) resulted in a 50% loss of biotinylated P2X₁ receptor on the cell surface. Even a 2-min treatment with $\alpha\beta$ meATP (10 μ M) was sufficient to cause a long-lasting inhibition of the contractile response. Cell surface receptors recovered within 10 min of terminating the agonist application, and the contractile response recovered more slowly. Therefore, sustained application of agonist to P2X₁ receptors results in 1) rapid (few milliseconds) channel opening, 2) fast desensitization ($\tau \sim$ 300 ms), and 3) receptor internalization ($\tau \sim$ 1–3 min). If the agonist application is terminated, the receptors reappear at the cell surface ($\tau \sim$ 10 min).

B. Homomeric P2X₂ Receptors

The rat P2X₂ receptor cDNA was isolated from a library constructed from NGF-differentiated PC12 cells by testing pools for functional expression in *Xenopus* oocytes (37). The human receptor cDNA was amplified from pituitary gland (292).

1. Agonists

The current elicited by ATP differs prominently from that observed at P2X₁ receptors in that the agonist action of ATP is not mimicked by $\alpha\beta$ meATP. There are no agonists currently known that are selective for P2X₂ receptors, but certain effects of ions are useful. Thus P2X₂ receptors are potentiated by protons (97, 244, 441, 500) and by low concentrations of zinc and copper (37, 500, 511). Systematic mutation of cysteine and histidine residues in the rat P2X₂ receptor has indicated that 2 of the 9 histidines (His-120, His-213) but none of the 10 cysteines seem to contribute to the binding of zinc (74). In contrast, the potentiation by protons was much reduced by removing a different histidine residue (His-319) (74).

Homomeric P2X₂ receptors have been thoroughly studied at the single-channel level after expression in oocytes and HEK293 cells (Fig. 6) (97–99). Several models were fitted to the kinetics of the single channels, and the most likely (Fig. 6) had the following features: 1) three molecules of ATP bind to the channel; 2) the binding steps are not independent, but positively cooperative; 3) two open states connect to a common ATP-independent closed state; 4) activation and inactivation proceed along the same pathway; and 4) channels only open when fully liganded.

Efforts have been made to identify amino acid residues that might contribute to the ATP binding site. On the basis that hydrogen bonding with polar or charged side chains were likely to be involved, such amino acids were mutated individually to alanine (217). A region was identified proximal to the first transmembrane domain that

contained two lysine residues that were critical for the action of ATP (Lys-69 and Lys-71); these correspond to the residues identified by Ennion et al. (116) in the P2X₁ receptor. Further analysis of this region showed that the attachment of negatively charged methanethiosulfonates to a cysteine introduced at Ile-67 resulted in a parallel rightward shift in the ATP concentration-effect curve, consistent with a reduced affinity for ATP. Positive or uncharged methanethiosulfonates depressed the maximal responses to ATP, consistent with an impairment of the conformational changes leading from binding to channel opening. This inhibition by the methanethiosulfonates was prevented by preexposure to ATP, suggesting occlusion of the binding site (217). Taken together, these results are consistent with Ile-67 being located close to the binding pocket for ATP.

2. Antagonists/blockers

There are no antagonists selective for P2X₂ receptors. The responses to brief applications of ATP are inhibited by calcium ions, with an IC₅₀ of ~5 mM (121), and it may be possible to take advantage of this to differentiate them from other forms. The divalent cations cause a fast (i.e., low affinity) block of single P2X₂ channels (98, 99). The order of potency is Mn > Mg > Ca > Ba, which is the order of ionic radii. This suggests that the divalent ions are binding to a charged site within the channel (98). In the case of calcium, the concentration giving 50% block was 3.8 mM. These observations correlate well with those made by Nakazawa and Hess (326) for PC12 cells.

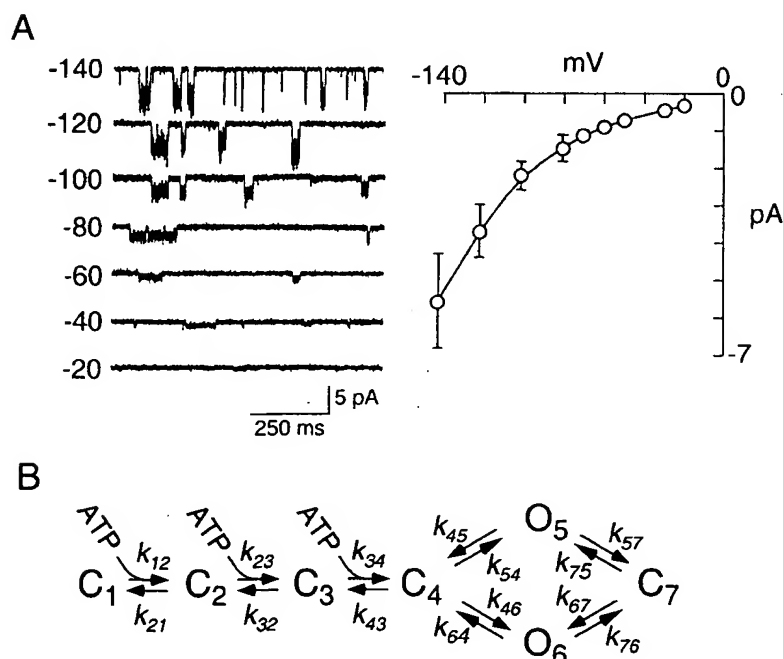


FIG. 6. Single-channel currents elicited by ATP in oocytes expressing P2X₂ receptors. *A*, left: typical unitary currents in response to ATP (2 μ M; 1 mM magnesium, 1 mM calcium) at the membrane potentials indicated. *Right*: current-voltage plot for unitary currents shows strong rectification; this was unchanged by removal of calcium and magnesium. Outside-out recordings from stably transfected HEK293 cell. *B*: kinetic scheme for gating of the P2X₂ receptor which best fits the single-channel records has three sequential ATP binding steps (C₁ to C₂, C₂ to C₃, and C₃ to C₄). [From Ding and Sachs (97). Reproduced from *Journal of General Physiology*, 1999, by copyright permission of The Rockefeller University Press.]

3. Permeation properties

A) SINGLE-CHANNEL RECORDING. Single-channel recordings made on outside-out patches from HEK293 cells expressing P2X₂ receptors have been described (97, 98). Openings were associated with an unusually large increase in current noise, suggestive of several open states interchanging more rapidly than could be resolved. The maximal probability of opening observed was 0.61; the EC₅₀ for ATP was ~10 μ M, and the Hill coefficient was 2.3. The unitary currents showed strong inward rectification and had a conductance of 30 pS at -100 mV (Fig. 6). Current flow through the channels was associated with excess current noise, which could not be accounted for by the flickery block of impermeant ions. The permeant ions are ordered in selectivity according to Eisenman's sequence IV ($K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$), and the channels were essentially impermeant to NMDG, Tris, and tetraethylammonium (TEA).

B) RECTIFICATION. At the whole cell level, the currents induced by ATP also show strong inward rectification (37, 122). This is very variable from cell to cell (oocytes or HEK293 cells, with occasional cells showing almost linear current-voltage relations (122). The rectification results in part from rectification in the unitary currents; unitary conductance falls from ~20 pS at -120 mV to ~10 pS at -50 mV. The mechanism of this rectification is not known; its persistence in divalent-free solutions indicates that it does not simply result from block of the permeation pathway by divalent cations (97, 98). Voltage-jump experiments indicate that there is an additional time-dependent component of inward rectification in the voltage range of -100 to -40 mV; when the membrane is stepped to -100 mV, the new conductance is reached with a time constant of ~12 ms (522).

C) CALCIUM PERMEABILITY. P2X₂ receptors are permeable to calcium. P_{Ca}/P_{Na} is ~2.5 in 5 mM external calcium; this is less than homomeric P2X₁ (122) and P2X₄ (145) receptors but more than homomeric P2X₃ receptors (482). Unfortunately, it is not straightforward to make an accurate measurement of the calcium permeability of the P2X₂ receptor. The preferred experiment, in which calcium is the only extracellular cation, is difficult because of the block of the current that this causes. The alternative approach is to combine extracellular calcium with another extracellular cation that is impermeant. NMDG is commonly used, but this can be complicated by the time-dependent increase in permeability to NMDG that occurs in some cells transfected with P2X₂ receptors (see below).

The calcium permeability has also been measured in receptors with mutations in the second membrane-spanning domain (308). P_{Ca}/P_{Cs} was reduced by about half when a hydrophobic residue (or tyrosine) replaced the polar side chains of Thr-336, Thr-339, and Ser-340. In general, the larger the volume of the side chain at Thr-339

or Ser-340, the smaller was P_{Ca}/P_{Cs} ; more significantly, the introduction of fixed negativity at this position (T339E) greatly increased the relative permeability to calcium. These findings are consistent with the model proposed on the basis of methanethiosulfonate accessibility, that residues in the region Thr-336 through Ser-340 are located in a narrow region of the permeation pathway (379).

D) CYSTEINE SUBSTITUTION. Amino acid residues that might contribute to the permeation path have been identified by the substituted cysteine accessibility method. Rassendren et al. (379) used three methanethiosulfonates to probe the region from Val-316 to Thr-354 in the rat P2X₂ receptor. They found that application of methanethiosulfonates inhibited the currents evoked by ATP in the cases of I328C, N333C, T336C, and D349C and augmented the current for S340C and G342C. In the case of L338C and D349C, only the small positively charged methanethiosulfonate [ethylammonium-methanethiosulfonate (MTSEA)] was effective; for D349C (but not L338C), this block required channel opening. Because MTSEA can permeate the open channel, it was suggested that Asp-349 lies on the internal side of the channel "gate." For the other three positions (I328C, N333C, and T336C), inhibition occurred with methanethiosulfonates that were negatively charged [sulfonatoethyl-methanethiosulfonate (MTSES)] or positively charged [ethyltrimethylammonium-methanethiosulfonate (MTSET)]. It was concluded that these residues lay outside the membrane electric field. On the other hand, the development of block by methanethiosulfonates at T336C introduced new rectification into the channel, which suggests that it might lie in the permeation path. These authors drew attention to the difficulties in using MTSEA, which gave much more variable results than MTSES and MTSET. Substitutions at Ile-328, Asn-333, and Thr-336 (with Ala, Gly, Asn, Asp, Glu, Lys, Ser, and Gln) also increase the dilation of the channel; all cells expressing N333A show a large increase in NMDG permeability and YO-PRO-1 uptake (481). The results of the substituted cysteine accessibility experiments are summarized schematically in Figure 7.

Egan et al. (109) carried out similar experiments, using ionic silver and MTSEA as the probes for reactive cysteines. Results with silver were complicated by a potentiation of currents at the wild-type receptor, presumably acting in a manner similar to zinc (see above); however, irreversible inhibition of the ATP-induced currents was observed for many mutants, including I328C, N333C, and T336C. In their study, S340C and D349C failed to express, and G342C showed irreversible potentiation. As in the experiments of Rassendren et al. (379), MTSEA (1 mM) produced variable inhibition; however, the most marked inhibitions (~40–50%) were seen with I328C, L334C, L338C, and T339C. Although T336C gave almost 100% inhibition by MTSET and MTSEA in the studies of Rassendren et al. (379), this mutant was unaffected by

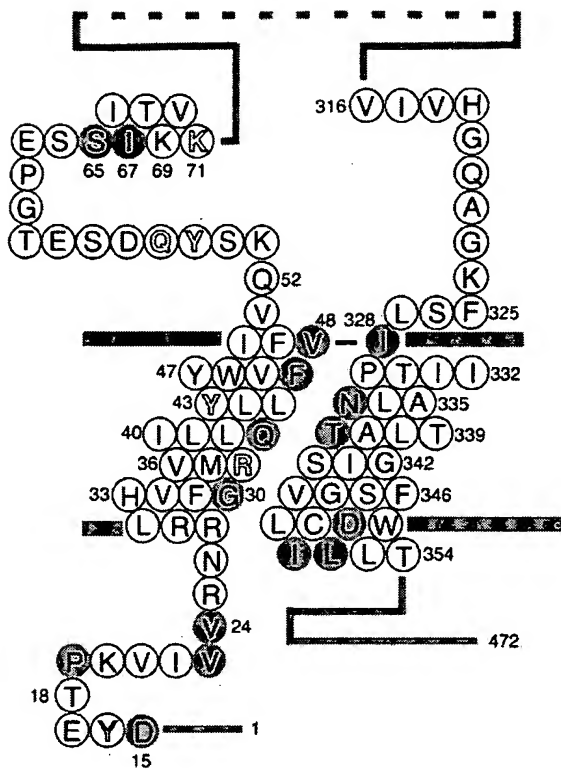


FIG. 7. Schematic summary of cysteine substitution studies on rat P2X₂ receptor. Two segments of the P2X₂ receptor are depicted; Asp-15 to Lys-71 includes the first transmembrane domain, and Val-316 to Thr-354 includes the second transmembrane domain. The representation of the membrane-spanning segments as α -helices is hypothetical and suggested only by secondary structure prediction algorithms. Each amino acid shown has been individually mutated to cysteine. Mutated receptors were expressed in HEK293 cells, and currents were elicited by ATP. Outline letters (Tyr-16, Arg-34, Tyr-43, Gln-56, Lys-71) indicate those positions where no functional channels were expressed. Solid gray circles indicate those positions where a methanethiosulfonate inhibited the current (including membrane-permeable methanethiosulfonates). The line between Val-48 and Ile-328 indicates the disulfide bond that forms when both residues are substituted by cysteine; these residues are not necessarily provided by the same subunit. [Data from Rassendren et al. (379), Jiang et al. (216), and Jiang et al. (217).]

MTSEA in the work of Egan et al. (109). The reasons for the differences at T336C are not completely obvious. Rassendren et al. (379) found that T336C reacted about five times more slowly to MTSEA than did I328C; it is possible that the short applications used by Egan et al. (109) were insufficient to observe inhibition with MTSEA, an interpretation consistent with their observation of rapid, substantial, and irreversible inhibition of T336C by silver. I328C and D349C were strongly inhibited by MTSEA in both studies, and Rassendren et al. (379) showed that MTSEA attachment had the fastest on rate at these positions. In the case of D349C, Egan et al. (109) observed inhibition only after coexpression with wild-type subunit, because ATP did not elicit currents at D349C mutants when expressed alone.

Coexpression in oocytes of wild-type channels with channels incorporating the T336C mutation indicates that the inhibition by MTSET is not a dominant phenotype. When the ratio of the wild-type to mutant subunits was systematically altered (by changing the ratio of the DNA or RNA injected), it was found that the degree of inhibition by MTSET depended simply on the fraction of mutant subunit expressed (442). In other words, if a channel is formed by three subunits, the attachment of MTSET to a single subunit causes only ~33% inhibition of the current. Concatenated cDNAs (up to 4 joined in series) encoding P2X₂ subunits have been made in which the T336C mutation was introduced into each one (or more) of the subunits (442). The inhibition by MTSET was proportional to the number of subunits in the construct that contained the T336C mutation (for dimers and trimers), consistent with a channel in which Thr-336 occupies a position near the external vestibule. When the construct was lengthened to four subunits, it was found that the inhibition by MTSET became dependent on the position in the order of four subunits at which the Thr-336C mutation was introduced. T336C in the fourth position gave little or no inhibition, suggesting that the fourth subunit did not contribute to channel formation. These experiments are therefore consistent with the biochemical studies described in section III C and suggest that a threefold assembly of subunits is a key contributor to the functional channel.

Cysteines have also been introduced individually into positions before, through, and after the first transmembrane domain (Gly-30 to Val-51); their accessibility was tested with a range of methanethiosulfonates (216). Introduction of cysteine at some positions, where the amino acid is highly conserved among all P2X receptors, led to nonfunctional channels; these were Tyr-16, Arg-34, Tyr-42, Tyr-55, and Gln-56 (see also Ref. 173). The methyl methanethiosulfonate (MTSM), which is small and uncharged, inhibited the currents (>60%) for the mutants D15C, P19C, V23C, V24C, G30C, Q37C, F44C, and V48C. The last four of these would be exposed along the same face of a helix (Fig. 7), but it is unlikely that they contribute directly to the lining of the aqueous pore. First, they are predominately large nonpolar residues and, second, the action of MTSM was mimicked by charged methanethiosulfonates only in the case of Val-48. Val-48 is located at the outer edge of the first transmembrane domain (Fig. 1). However, the inhibition by MTSM (and MTSES and MTSET) in the case of at V48C was greater when the channel was opened by ATP application than when it was not. This suggests that channel opening involves the movement of Val-48 into a position where it reacts more readily with methanethiosulfonates. Consistent with this interpretation was the direct demonstration that ATP does not open the channel in which the V48C mutation is combined with I328C, but ATP becomes effective after treatment with a reducing agent. This indicates that a

disulfide bond can form between these two residues and shows that a separation of these residues is an essential component of channel opening (216); the studies do not indicate whether the two cysteines (V48C and I328C) are on the same or different subunits. A further surprising finding of this study was that the point mutation F44C appeared to move the channel conformation in favor of the open state(s). ATP was more effective (EC_{50} changed from 10 to $<1 \mu\text{M}$), $\alpha\beta\text{meATP}$ became an effective agonist (EC_{50} changed from $>300 \mu\text{M}$ to $10 \mu\text{M}$), and the whole cell current declined more slowly on wash out of agonist. Phe-44 would be positioned one turn of a helix from Val-48 (Fig. 7), so the results are consistent with (outward) movement of this part of the molecule being a critical component of channel opening.

Silver has also been used as probe of cysteines in the first transmembrane domain (173). These experiments are again difficult to interpret because 1) the short duration of application ($<10 \text{ s}$) may not be sufficient for thiolation to proceed to steady state with 500 nM silver, and 2) silver itself caused a transient potentiation of the current even in wild-type cells. Overall, these experiments also fail to provide evidence that any of the positions in this region are exposed to the aqueous ion conducting pathway, although reaction with cysteines at the ends of the transmembrane domain (H33C and I50C) significantly but incompletely (40–50%) reduced the currents evoked by ATP. Silver modification of K53C and S54C, which are located just outside the first transmembrane domain, reduced the peak current evoked by ATP by $\sim 50\%$ without change in the EC_{50} .

E) PERMEABILITY INCREASE WITH TIME. In some cells expressing $P2X_2$ receptors, the permeation pathway of the $P2X_2$ receptor appears to dilate during agonist applications lasting for several seconds (HEK293 cells, Refs. 481, 480; oocytes, Ref. 229). This is evidenced by a progressive increase in the permeability to large organic cations, including NMDG, Tris, and TEA (Figs. 8 and 9). Measured under bi-ionic conditions in mammalian cells, the permeability to NMDG is initially very low ($<5\%$ that of sodium), but this increases (exponentially with time constant 7 s) until NMDG is $\sim 50\%$ as permeable as sodium (480, 481).

The concentrations of ATP that elicit the permeability increase are similar to those required to activate the initial current, and the forward rate into the increased permeability state is linearly related to the ATP concentration ($k_1 = 3 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$). In contrast, the apparent first-order rate constant for opening of the NMDG-impermeable channel under similar whole cell recording conditions is about three orders of magnitude faster (R. J. Evans and R. A. North, unpublished observations), which is about the same as the estimates from single-channel kinetics (97; see Fig. 6; $k_{12} = 3$; $k_{23} = 20$, $k_{34} = 24 \mu\text{M}^{-1}\text{s}^{-1}$). The permeability increase in homomeric $P2X_2$ receptors was enhanced by some mutations thought to be in the pore-forming region on the basis of cysteine-scanning mutagenesis (e.g., N333A; Ref. 481).

One difficulty in interpreting the dilation experiments is that they are necessarily carried out in sodium-free external solutions, and this itself could be responsible for the behavior. Evidence against this interpretation was provided by studies carried out in physiological solutions,

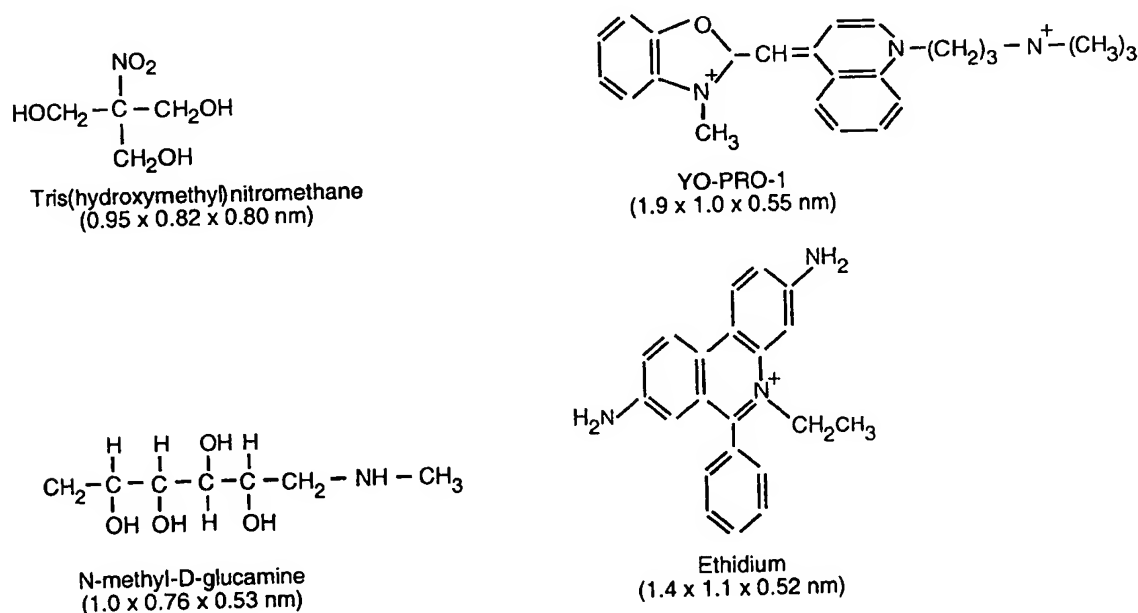


FIG. 8. Structures and dimensions of some cations used to estimate the pore size of $P2X$ receptors. These dimensions were measured from space-filling models (van der Waals radii) of energy-minimized conformations drawn in CSC ChemDraw.

in which case the dilatation was followed by the entry of a trace amount (1 μ M) of the fluorescent propidium dye YO-PRO-1. At 100 μ M ATP, the increase in YO-PRO-1 fluorescence occurs exponentially with a time constant of ~ 7 s, which is the same as the value obtained for the increase in NMDG permeability (480, 481). The dimensions of NMDG are somewhat smaller than those of YO-PRO-1 (480) (Fig. 8). This puts a lower limit on the size of the dilated channel; the upper limit is not known. It is known, however, that when the agonist is removed, the dilated channel reverts within 2 s to its closed state. The dilation of the channel is not observed in all cells (typically $\sim 40\%$ with transient transfection, 20% with stably transfected cells) (481); such variability suggests the possibility that the behavior might result from the involvement of yet unidentified interacting proteins.

4. Desensitization/inactivation

With whole cell recording, currents at P2X₂ receptors decline little during agonist applications of a few seconds (37, 81) (Fig. 5). For this reason, the P2X₂ receptor is generally described as nondesensitizing, compared with the P2X₁ and P2X₃ receptors. However, there is a progressive decline in the current that occurs during applications of several tens of seconds (slow desensitization; Fig. 5). This has been investigated in two respects: 1) by mutagenesis and 2) by studies on its calcium dependence. Amino acid residues in the NH₂ terminus, the transmembrane domains, and the COOH terminus can influence this slow desensitization.

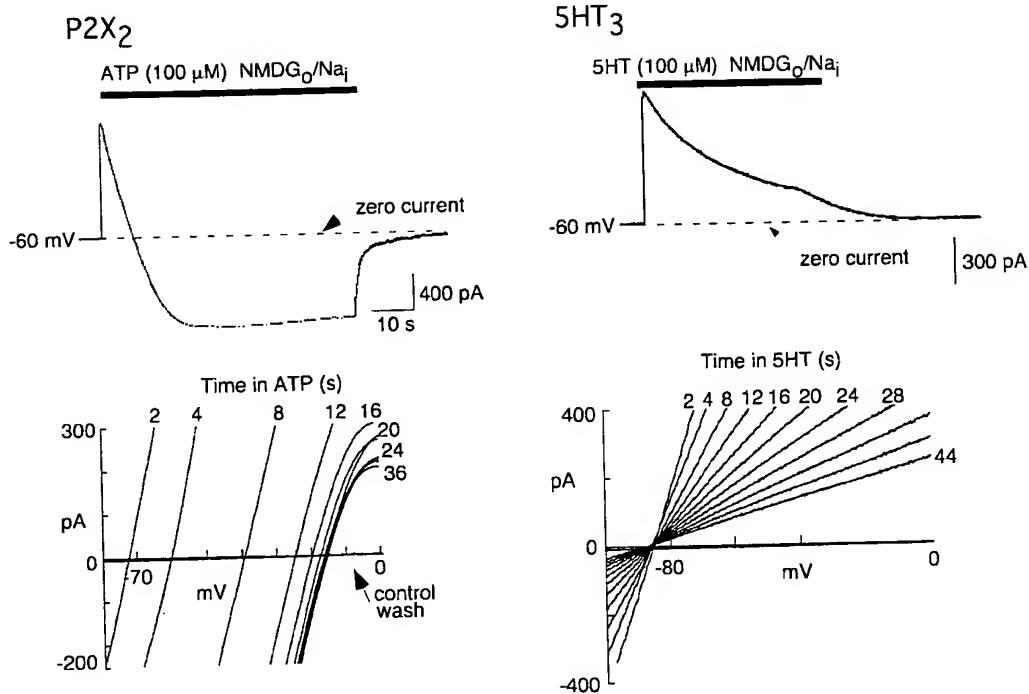
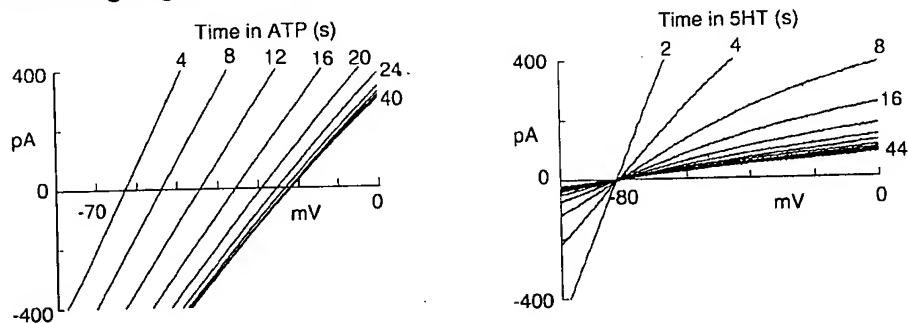
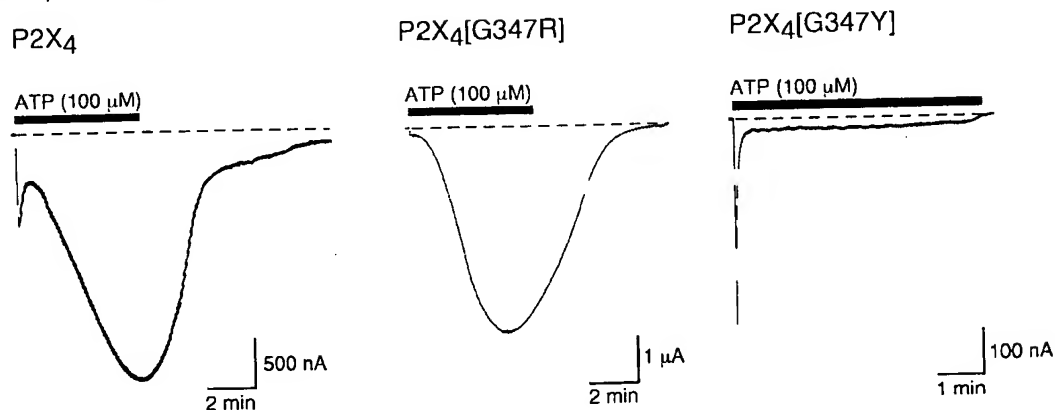
In the NH₂ terminus, Thr-18 can be phosphorylated by protein kinase C (33). The mutants T18A or T18N show much accelerated slow desensitization; this is complete within 1–2 s, which is still considerably slower than the rate of fast desensitization observed for homomeric P2X₁ and P2X₃ receptors. A similar effect was observed with K20T, which removes the consensus site for protein kinase C phosphorylation while leaving the conserved threonine unchanged. These results suggest that the wild-type channel is constitutively phosphorylated by protein kinase C, and when this does not occur, the channel exhibits more rapid desensitization (33). However, it is not clear whether this explanation can be generalized among P2X receptors. Threonine occupies the position corresponding to Thr-18 in all P2X receptors. P2X₁ receptors exhibit fast desensitization, and this becomes even faster for P2X₁[T18A] (115); however, P2X₁ receptor desensitization is unaltered by phorbol esters (495). P2X₃ receptors with the corresponding mutation do not express functional currents (364).

As for the COOH terminus, it is known that the splice variant of the rat P2X₂ receptor with a shortened COOH terminus (P2X_{2b}; missing the 69 amino acids from Val-370 to Gln-438 inclusive) shows a rather faster current decay

(time constant ~ 24 s) than the wild-type receptor (time constant ~ 111 s) (rP2X_{2a}) (40, 418, 421). This difference, some fourfold, is not seen for the human receptors (292). The additional amino acids found in P2X_{2a} compared with rP2X_{2b} begin with Val-370; the last hydrophobic acid of the second membrane-spanning domain is Leu-353. The rat P2X₂ receptor truncated so as to end at Val-370 desensitizes with intermediate time constant when expressed in oocytes (~ 60 s; Ref. 421). However, the valine is critical because the receptor truncated at Lys-369 desensitizes very much faster (< 1 s). Smith et al. (421) identified other residues in the segment of the P2X receptor beginning with Val-370 (Val-Arg-Thr-Pro-Lys-His-Pro in P2X_{2a}) as being important in desensitization. This is generally consistent with results from Koshimizu and colleagues (255–257) using whole cell calcium measurements as the assay for P2X receptor activation. They studied the changes in intracellular calcium elicited by ATP in GT1 cells expressing P2X₂ receptors and found that positively charged residues in this segment played a role in determining the kinetics of desensitization. Zhou et al. (522) found that certain substitutions at Asp-349, near the inner border of the second transmembrane domain, can also accelerate desensitization. It has been suggested that its negatively charged side chain might interact with the positive charges following Val-370 to stabilize a long-lived channel open state (256, 257, 522). One might equally speculate that an attached phosphate group at Thr-18 interacts with these positive charges.

The role of Ser-431 has also been studied (66); this is situated within the region that is spliced out in the P2X_{2b} form. The residue is situated at a protein kinase A consensus site, and introduction of the catalytic subunit of protein kinase A into the cytoplasm of HEK293 cells expressing the P2X₂ receptor led to an inhibition of the ATP-evoked currents. The effect was not seen in the S431C receptor. The inhibition was associated with an increased rate of desensitization. In the experiments of Werner et al. (495) (see sect. IV A4), chimeras were made between the P2X₁ and P2X₂ subunits. To make the P2X₂ receptor desensitize as rapidly as the P2X₁ receptor, it was necessary to provide it with both segments 14–47 and 332–365 of the P2X₁ receptor. These sequences include Thr-18 (in P2X₁ and P2X₂), but they do not include Lys-369 (P2X₂, corresponds to Lys-370 in P2X₁).

The calcium dependence of the decline in the current during the application of ATP was studied by Ding and Sachs (99). In whole cell recording mode, currents decline almost linearly with time; they reach half their initial amplitude in ~ 2 min. This decline was not seen in calcium-free external solution. In outside-out patches, currents at P2X₂ receptors decline much more rapidly than in whole cell configuration; with normal extracellular calcium (1 mM) this decline occurs within tens of milliseconds (99, 521). This basic observation implies that the

A HEK293 cells**B** Nodose ganglion neurons**C** *Xenopus* oocytes

decline of the current is prevented in the whole cell configuration because of the presence of some intracellular modulator, which is lost slowly in the whole cell recording but lost rapidly in outside-out patches (99). On the other hand, it is extracellular calcium that plays the key role in the decline of the current. Ding and Sachs (99) term this decline inactivation (i.e., inactivation by calcium) rather than desensitization (which may imply involvement of only the receptor protein and the ligand ATP). In the promotion of inactivation, calcium is better than magnesium, barium, and manganese (EC_{50} values are respectively 1, 2, 3, and 5 mM). The maximum rate of decline of the ATP-induced current, observed with 2.5 mM calcium, is 40 s^{-1} (corresponding to a time constant of 25 ms). The decline of the current (inactivation) is steeply dependent on the ATP concentration (EC_{50} 19 μM , Hill coefficient 2.8), the calcium concentration (EC_{50} 1.3 mM, Hill coefficient 4.0), and membrane potential (inactivation was faster with hyperpolarization, changing e -fold for 26 mV in potential) (99).

In summary, extracellular divalent cations have (at least) two distinct actions on the homomeric P2X₂ receptor. First, they block the open channel; in this case the EC_{50} for calcium is ~ 5 mM, the order of effectiveness is $\text{Mn} > \text{Mg} > \text{Ca} > \text{Ba}$, and the results fit well to a single binding site. Second, they reduce the probability of a channel being open; in this case they bind to the liganded channel, the EC_{50} for calcium is about 1.3 mM, the order of effectiveness is $\text{Ca} > \text{Mg} > \text{Ba} > \text{Mn}$, and the results are best fit by the binding of four Ca ions.

ATP currents increase in size with repeated applications in the case of hippocampal neurons expressing heterologous P2X₂ receptors. Khakh et al. (235) used Sindbis virus to infect neonatal hippocampal neurons in culture with a P2X₂-GFP construct. The cells responded to ATP with currents typical of P2X₂ receptors in other expression systems, but these currents doubled in amplitude when ATP was applied repetitively at 1 Hz. This increase was correlated with a redistribution of the receptor, as visualized by its GFP tag, over distances of several micrometers into varicose "hot spots." The redistribution was not seen with the T18A mutant receptor, suggesting

that it might result from activity-dependent phosphorylation by protein kinase C.

5. Interaction with nicotinic acetylcholine receptors

When oocytes are injected with RNAs encoding P2X₂ receptors, and also the α_3 - and β_4 -subunits of nicotinic receptors, they show responses to both ATP and acetylcholine; these can be selectively antagonized with appropriate receptor blockers (237). However, with concomitant application of both agonists, the resultant current is less than the expected sum of the two independent currents. A similar observation had been made previously in several native cells (see sect. vE5). Such occlusion of the currents indicates an interaction between the two receptors. It was more marked when the channels were expressed at high levels and was not seen in oocytes injected with lower amounts of RNAs. This might suggest the need to generate critical amounts of a signaling molecule for the interaction to occur.

C. Homomeric P2X₃ Receptors

P2X₃ receptor subunit cDNAs were isolated from rat dorsal root ganglion cDNA libraries (60, 274), from a human heart cDNA library (147), and from a zebrafish library (32, 108).

1. Agonist actions

The mimicry of ATP by $\alpha\beta\text{meATP}$ makes these receptors similar to P2X₁ and distinct from the other homomeric forms. 2-Methylthio-ATP (2-MeSATP) is as potent as (274) or more potent than (60, 147) ATP at P2X₃ receptors. Diadenosine pentaphosphate (Ap5A) is a full agonist, as measured by calcium fluxes in transfected 1321N1 human astrocytoma cells (25). The actions of ATP are potentiated by zinc (rat P2X₃: $EC_{50} \sim 10\text{ }\mu\text{M}$) (501) and cibacron blue (human P2X₃: EC_{50} 3 μM). Diadenosine triphosphate (Ap3A) is more potent than at P2X₁ receptors (499), whereas $\beta\gamma\text{meATP}$ is strikingly less so (60, 147). The zebrafish receptor is notably less sensitive to $\alpha\beta\text{meATP}$ than the rat and human counterparts (32, 108).

FIG. 9. Time-dependent permeability increase in cells expressing P2X₂ and P2X₄ receptors. A: HEK293 cells expressing P2X₂ receptors (left) and serotonin (5-HT₃) receptors (right). In each case, the only external cation was *N*-methyl-D-glucamine (NMDG); the internal solution was sodium. At -60 mV, the application of agonist elicits an outward current, reflecting the low permeability to NMDG. In the case of the P2X₂ receptor, the current turns inward within a few seconds, and this is accompanied by a positive shift in reversal potential (A, bottom panels). In the case of the 5-HT₃ receptor, the current declines (due to desensitization) but never becomes inward; the reversal potential does not change. B: nodose ganglion neurons show responses to ATP (100 μM) and 5-HT (30 μM) that closely resemble those seen in transfected HEK293 cells. C: *Xenopus* oocytes expressing wild-type P2X₄ receptors show a biphasic response to ATP (left). The initial transient current reflects current flow through a sodium-selective channel, and the later, larger, and more prolonged current reflects current flow through an NMDG-permeable channel (see Ref. 229). Mutation of a glycine residue in the second membrane-spanning domain selectively eliminates either the fast, sodium-selective current (G347R) or the sustained NMDG-permeable component (G347Y). [A and B modified from Virginio et al. (481); C modified from Khakh et al. (229).]

2. Antagonists/blockers

The antagonists suramin, PPADS, and TNP-ATP do not readily distinguish between P2X₁ and P2X₃ receptors, but NF023 is ~20 times less effective at P2X₃ than P2X₁ receptors. Protons inhibit currents at rat P2X₃ receptors, with an EC₅₀ of ~1 μ M (pK_a 6). The P2X₃ receptor is remarkably insensitive to block by extracellular calcium (EC₅₀ ~90 mM) (482).

3. Permeation properties

Rat P2X₃ receptors are cation-selective channels (274). The relative permeability of calcium to sodium (P_{Ca}/P_{Na}) is ~1.2 (in 5 mM calcium, NMDG solution) (482).

4. Desensitization/inactivation

At low concentrations (30–300 nM), ATP elicits currents that are sustained for several seconds, but with higher concentrations the currents show prominent desensitization (Fig. 5). The desensitization occurs with a time constant of <100 ms at concentrations of 30 μ M ATP (274). As for P2X₁ receptors, recovery from this desensitization is very slow, and reproducible responses to ATP (or $\alpha\beta$ meATP) can only be obtained when applications are separated by at least 15 min.

Cook, McCleskey, and colleagues (82, 83) found that recovery from desensitization can be greatly accelerated by increasing the extracellular calcium concentration. The time constant for recovery was 7 min at 1 mM calcium and 3.5 min at 10 mM; gadolinium had a similar accelerating effect at 10 μ M. This effect of calcium was related to the period of time for which the concentration was elevated and occurred whether or not the calcium concentration was increased at the same time that ATP was applied. Indeed, an elevation of calcium concentration was effective to accelerate recovery from desensiti-

zation even when it was applied several minutes before the next application of ATP. This suggests that calcium and gadolinium can bind to a desensitized form of the channel and accelerate its recovery into a nondesensitized, closed state.

D. Heteromeric P2X_{2/3} Receptors

In certain sensory neurons, sympathetic ganglion cells, and brain neurons, the action of ATP is mimicked by $\alpha\beta$ meATP, but there is no desensitization in the millisecond time scale (445). This type of response is mimicked by coexpression of P2X₂ and P2X₃ receptors (274). Direct association between the subunits has been shown by coimmunoprecipitation after expression in insect cells using baculovirus expression (374, 462).

1. Agonists

There are potential difficulties in interpreting the results of functional studies on cells expressing two or more subunits when each can make the homomeric channels, because it must be assumed that the cell assembles the homomeric as well as heteromeric channels. The isolation of heteromeric channels is relatively straightforward in the case of the P2X_{2/3} heteromer because homomeric P2X₂ receptors are not activated by $\alpha\beta$ meATP, and currents at homomeric P2X₃ receptors rapidly desensitize and rundown with repeated applications. Therefore, P2X_{2/3} heteromeric channels can be defined on the basis of a sustained current elicited by $\alpha\beta$ meATP repeated at intervals of <5 min. P2X_{2/3} heteromeric channels share some properties with homomeric P2X₂ receptors; they are potentiated by low pH, and they do not desensitize within the time course of a few seconds (Table 5). Ap5A has little agonist action at either homomeric P2X₂ receptors or heteromeric P2X_{2/3} homomeric receptors, even though it

TABLE 5. Heteromeric P2X_{2/3} receptors take some properties from the P2X₂ subunit and others from the P2X₃ subunit

	P2X ₂	P2X ₃	P2X _{2/3}	Reference Nos.
<i>P2X_{2/3} resembles homomeric P2X₃</i>				
$\alpha\beta$ -Methylene ATP (EC ₅₀)	>300 μ M	~1 μ M	~1 μ M	274
Ap5A (EC ₅₀)	Inactive	~1 μ M	~1 μ M	287
TNP-ATP (IC ₅₀)	>1 μ M	~1 nM	~3 nM	483
Zinc (100 μ M)	Increase 1,300%	Decrease 17%	Increase 90%	287
<i>P2X_{2/3} resembles homomeric P2X₂</i>				
Ip5I (IC ₅₀)	Inactive	~3 μ M	Inactive	287
Desensitization (τ)	>10 s	<100 ms	>10 s	274
Calcium block (IC ₅₀)	~5 mM	100 mM	10 mM	482
Acidification (pH 6.3)	Potentiate	Inhibit	Potentiate	287, 442

Ap5A, diadenosine pentaphosphate; TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl)-ATP; Ip5I, di-inosine pentaphosphate; Ap5A, diadenosine pentaphosphate.

activates homomeric P2X₃ receptors in parallel experiments (25).

2. Antagonists/blockers

The P2X_{2/3} heteromer shares with the homomeric P2X₃ the high sensitivity to block by TNP-ATP (455, 483), as well as PPADS and suramin (48, 435) (Table 5). The high affinity for TNP-ATP results rather from a fast association rate rather than a slow dissociation rate (435). Ip5I is much more potent to block P2X₁ and P2X₃ homomers (242) than to block the P2X_{2/3} heteromers and is therefore useful to distinguish between P2X₃ and P2X_{2/3} receptors (103, 287; Table 5). Increasing the concentration of calcium ions also inhibits currents through P2X_{2/3} receptors, but they are less sensitive in this regard than P2X₂ homomers (482).

3. Permeation properties

The calcium permeability of the receptor is close to that of the P2X₃ subunit (P_{Ca}/P_{Na} 1.2–1.5; Ref. 482). A time-dependent increase in NMDG permeability can also occur in P2X_{2/3} heteromeric channels (229).

4. Desensitization/inactivation

The relatively slow desensitization of currents through heterologously expressed P2X_{2/3} heteromers is one of its defining features. However, this has not been studied in detail, and the regions of the two subunits involved are not determined.

In summary, with respect to each of its main properties, the P2X_{2/3} receptor closely resembles homomeric P2X₂ receptors in certain ways and homomeric P2X₃ receptors in others (Table 5).

E. Homomeric P2X₄ Receptors

Five groups independently isolated cDNAs for the rat P2X₄ receptor. These were from superior cervical ganglion (44), brain (412, 430; named P2X₃ in this paper), hippocampus (29), and pancreatic islet cells (491). Human (96, 145), mouse (464), chick cDNA (393), and *Xenopus* (222) cDNAs have also been isolated.

1. Agonists

Homomeric P2X₄ receptors are activated by ATP but not by α BmeATP. The most useful distinguishing feature of ATP-evoked currents at P2X₄ receptors is their potentiation by ivermectin; ivermectin does not potentiate currents in cell-expressing homomeric P2X₂, P2X₃, or P2X₇ receptors or P2X_{2/3} heteromers (234). It does, however, have a similar potentiating action at α 7-nicotinic acetylcholine receptors (258). Cibacron blue also potentiates

currents at the P2X₄ receptor, but not those at P2X₂ receptors (309); however, the effects are smaller than those seen with ivermectin. The currents can also be differentiated from those at P2X₂ receptors by the actions of copper and zinc. Both zinc and copper (10–100 μ M) potentiate P2X₂ receptor currents; however, zinc but not copper is effective at P2X₄ receptors (511). Acidification reduces currents at P2X₄ receptors but increases currents at P2X₂ receptors (441, 502). The inhibition of the current results from protonation of His-286, because it does not occur when this histidine residue is mutated to alanine (73). Histidine is found at this position only in the P2X₄ subunit.

2. Antagonists/blockers

The rat P2X₄ receptor is unusual among the P2X receptors in its relative insensitivity to blockade by the conventional antagonists suramin and PPADS (44, 430). Antagonism by PPADS at P2X receptors develops over several minutes and reverses only partially with a 20- to 30-min washing. This suggests that it might result from interaction between the aldehyde moiety of the pyridoxal ring and a lysine residue of the receptor (44). Buell et al. (44) identified one such candidate lysine in the rP2X₂ receptor; when this was replaced by glutamate (K246E), the inhibition by PPADS reversed fully within a 10-min washing. The P2X₄ receptor lacks the lysine at the equivalent position, but when lysine was introduced by mutagenesis (P2X₄-E249K), PPADS causes an almost irreversible inhibition (44). The hP2X₄ receptor is more sensitive to block by PPADS than the rat P2X₄ receptor; a domain stretching from Arg-83 to Glu-183 of the receptors was deemed to include the main determinant of PPADS sensitivity from experiments with a series of chimeric receptors (145). According to Jones et al. (221), the mouse P2X₄ receptor is blocked by PPADS (IC₅₀ ~10 μ M), whereas Townsend-Nicholson et al. (464) report that currents evoked by ATP at the mouse P2X₄ receptor are actually increased by PPADS.

Suramin also differs in its potency to block at the rat and human receptors (145, 430). In this case, the difference was largely accounted for by a single amino acid difference. The rat receptor has glutamine at position 78 and is relatively insensitive to suramin; the human receptor has lysine and is more readily blocked. The mouse sequence has glutamine in this position; ATP-evoked currents here are increased by concentrations of suramin (3–100 μ M) that block other P2X receptors (464) or are unaffected (221). A small potentiation by lower suramin concentrations was found at the rat P2X₄ receptor by Buell et al. (44). Suramin has six negatively charged sulfonate groups, and it is likely that minor differences in the disposition of positively charged side chains on the receptor may account for these phenotypic differences. Indeed,

because the inhibition by suramin (and PPADS and many of the related dyes) is allosteric rather than competitive, it is easy to imagine that the main determinants of its binding (which presumably include some positively charged amino acid side chains) might be quite different among the different P2X receptors. On the other hand, experimental conditions and protocols rather than the amino acid differences must underlie the conflict between the results of Jones et al. (221) and Townsend-Nicholson et al. (464).

Single-channel recordings from COS (119) or HEK293 (339) cells expressing P2X₄ receptors show channels with a unitary conductance of ~9 pS (at -100 mV). These currents are inhibited by magnesium (2–10 mM) in two ways: 1) the current amplitudes are reduced (implying a fast channel block) and 2) the mean open times are reduced (indicating an effect on ion gating). Although much more limited in scope, the results are broadly similar to those for P2X₂ channels (97, 98).

3. Permeation properties

When the application of ATP is of short duration, P2X₄ receptors operate as cation-selective channels; the calcium permeability is relatively high (4.2 in 8 mM calcium and NMDG, Ref. 430; 4.2 in 110 mM calcium, Ref. 44). In the human P2X₄ receptor, calcium contributes ~8% of the total inward current under normal conditions (1.8 mM extracellular calcium) (145).

When the application of ATP is continued for several seconds, the P2X₄ receptor channel becomes increasingly permeable to larger organic cations such as NMDG (229, 481). The phenomenon is essentially the same as that described above for the P2X₂ receptor; as for those receptors, it is observed in only a proportion (40–50%) of cells (229, 481). The main difference from the results with P2X₂ receptors is that the two components of the current (NMDG impermeable and NMDG permeable) are clearly separated in time (Fig. 9C). This appears to be because 1) the current through the NMDG-impermeable channel (*I*₁ in Ref. 229) desensitizes more quickly than that in the P2X₂ receptor, and, more importantly, 2) the time course of development of the NMDG-permeable (*I*₂) form is slower for the P2X₄ receptor (typically 50–100 s at 100 μM ATP) than for the P2X₂ receptor (5–10 s) (229, 481). Certain procedures allow the distinction of the two states of the receptor. For example, the *I*₂ state of the P2X₄ receptor does not occur if the extracellular calcium concentration is raised to 5 mM. Moreover, mutations of a glycine residue in the second transmembrane domain (Gly-347 in P2X₄) can produce receptors that exhibit only the *I*₂ form (G347R and G347K) or only the *I*₁ form (G347Y) (Fig. 9) (229).

It is tempting to speculate that the progressive development of an NMDG-permeable state results from con-

formational changes in the ion-conducting pathway. Given the relative sizes of a sodium ion, and NMDG or YO-PRO-1 (Fig. 8), such a conformational change could be quite minor. On the other hand, it must be kept in mind that the time-dependent increase in permeability is not seen in all cells; this suggests that other constituents of the expression system may be critical. Thus one possibility is that the NMDG permeation pathway is provided by a distinct membrane protein that is activated by the P2X receptor. These issues are discussed further in section IVJ3 with respect to the properties of P2X₇ receptors.

4. Desensitization/inactivation

Desensitization at P2X₄ receptors is intermediate between that observed at P2X₁ and P2X₂. There have been few systematic studies, but currents typically decline within 5–10 s at maximal ATP concentrations (100 μM) (145, 412, 491) (Fig. 5). Ivermectin greatly prolongs the action of ATP at P2X₄ receptors (234).

F. Homomeric P2X₅ Receptors

The P2X₅ receptor cDNA was first isolated from cDNA libraries constructed from rat celiac ganglion (81) and heart (146). A P2X receptor was also cloned from embryonic chick skeletal muscle and named P2X₈ (28); detailed comparison of the amino acid sequence of the ectodomain of this chick receptor with other P2X receptors indicates that it actually corresponds to the chick P2X₅ receptor. The same cDNA was more recently isolated by Ruppelt et al. (394); their paper also reports the genomic structure, which is completely conserved with other P2X receptors (and mouse P2X₅; Ref. 89). A bullfrog P2X₅ receptor has also been isolated from larval skin (named fP2X₅; Ref. 214). The only human cDNAs reported are missing exon 10 (hP2X_{5a}) or exons 3 and 10 (P2X_{5b}), and efforts to amplify a "full-length" cDNA, including exon 10, were unsuccessful (271). However, a sequence that corresponds to exon 10 can be found in the unordered human genomic sequences (AF168787). The translation is Ala-325-Gly-Lys-Phe-Ser-Ile-Ile-Pro-Thr-Ala-Ile-Asn-Val-Gly-Ser-Gly-Val-Ala-Leu-Met-Gly-Ala, which has three conservative amino acid differences from the equivalent rat sequence (see Fig. 1). Séguéla and colleagues (271) cloned a fragment of human P2X₅ corresponding to exons 1–9; this aligns with P2X receptors as far as Lys-327 (same number for human P2X₅ or rat P2X₅), just before the second transmembrane domain (Fig. 1). Comparison between the chicken, rat, and human sequences shows that they are closely related up to and including Arg-377 (rat P2X₅), but then diverge. This residue is ~18 amino acids toward the COOH terminus from the inner end of the second transmembrane domain and, by alignment with human genomic sequences, this corresponds to the

end of exon 11. This suggests species-specific splicing at this site, although the sequence corresponding to the rat COOH terminus (beginning at Gly-378) has no homologs in the human genomic database.

The most striking feature of the currents elicited by ATP in cells expressing the rat P2X₅ receptor is their small amplitude, compared with the currents observed with P2X₁, P2X₂, P2X₃, or P2X₄ receptors expressed under similar conditions. Maximal currents are typically 50–200 pA when expressed in HEK cells, whereas currents at rat P2X₂ receptors expressed under similar conditions are often several nanoamperes in amplitude. The currents otherwise resemble those seen at P2X₂ receptors: they show little desensitization, are not activated by $\alpha\beta$ meATP, and are blocked by suramin and PPADS at concentrations similar to those effective at P2X₂ receptors (81, 146).

Lê et al. (271) made a chimera between the human form (to the end of exon 9, amino acids Met-1 to Gly-328) and the COOH terminus of the rat P2X₅ receptor (h-rP2X₅). This was expressed in oocytes and resulted in currents that were activated by ATP, but which declined completely during a 2-s application of ATP (100 μ M). Repeated applications of ATP at intervals of several minutes had much smaller effects. This difference between the behaviors of the rP2X₅ receptor and the h-rP2X₅ suggest that residues in the NH₂ terminus and/or ectodomain also play a role in the shaping the kinetics of the response to ATP.

In contrast to the small currents observed with rat P2X₅ receptors, the chick P2X₅ expresses robustly in oocytes (28) and HEK293 cells (394). The chick P2X₅ receptor has some strikingly different properties compared with other P2X receptors. First, the channel has a relatively high permeability to chloride ions ($P_{Cl}/P_{Cs} = 0.5$). Second, the currents show desensitization at -60 mV ($\tau \sim 5$ s) but not at $+40$ mV; the desensitization at -60 mV largely disappears in low (0.1 mM) extracellular calcium. Third, $\alpha\beta$ meATP activates the receptor (equi-effective concentrations were ~ 10 -fold higher than for ATP) (394). The chloride permeability is of particular interest in view of reports that the current induced by ATP in developing chick skeletal muscle is similar (456). P2X₅ mRNA is well expressed by developing skeletal muscle (306).

G. Heteromeric P2X_{1/5} Receptors

P2X₁ and P2X₅ subunits can be coimmunoprecipitated (270, 462), and four papers report the properties of heteromeric P2X_{1/5} receptors in oocytes (270) or in HEK (172, 447, 463), COS-7, and CHO cells (172). The defining phenotype of the heteromer is a sustained current evoked by $\alpha\beta$ meATP, which is not seen for either of the homomers when expressed separately.

1. Agonists

Cells expressing the heteromeric receptor provide responses to ATP that have several unique features (172, 447, 463). First, they are more sensitive to ATP than those with homomeric receptors; concentrations as low as 3 or 10 nM evoke measurable currents. 2-MeSATP gives a maximal current similar to that of ATP, whereas $\alpha\beta$ meATP, adenosine 5'-O-(3-thiotriphosphate) (ATP γ S), and $\beta\gamma$ meATP produce only $\sim 80\%$ of the maximal current. Although they are more sensitive to ATP, the heteromeric receptors are not more sensitive than P2X₁ homomers to $\alpha\beta$ meATP. The dose-response curves for ATP and $\alpha\beta$ meATP have Hill slopes close to 1; for other receptors they are closer to 2. Second, the kinetics of the response are distinct; at the very low concentrations, the currents are sustained over several seconds but when the concentration exceeds 300 nM they show an initial peak that declines and is followed by a sustained component. At these concentrations, there is often a "rebound" increase in current when the agonist application is discontinued, as would be expected if the channel is passing from a desensitized state, through an open state, to the closed state. Third, repeated applications of agonist at intervals of 10 s give quite reproducible inward currents; in contrast, at the homomeric P2X₁ receptor, currents disappear when the agonist is reapplied at intervals less than several minutes.

2. Antagonists/blockers

Currents are inhibited by either an increase or a decrease of the extracellular pH (447). They are little affected by increasing the extracellular calcium concentration to 30 mM; this is similar to the P2X₁ homomer and different from the P2X₅ homomer (172). The sensitivity to suramin and PPADS is similar to that of each of the constituent homomers, but low concentrations of PPADS (100 nM) also potentiate the "plateau" phase of the current. However, the sensitivity to TNP-ATP (IC₅₀ 720 nM) is intermediate between the sensitive homomeric P2X₁ receptor (~ 1 nM) and the insensitive homomeric P2X₅ receptor (IC₅₀ > 10 μ M) (447).

3. Permeation properties

The P2X_{1/5} receptor is much less permeable to calcium (in bi-ionic solutions: P_{Ca}/P_{Na} 1.1) than the P2X₁ homomer (P_{Ca}/P_{Na} 3.9) (447). The calcium permeability of the P2X₅ homomer has not been measured. The NMDG permeability of the receptor is similar to that seen for P2X₂ or P2X₄ receptors ($P_{NMDG}/P_{Na} \sim 0.08$), and no increase in this permeability was observed during agonist applications of up to 20 s (447).

4. Desensitization/inactivation

At low concentrations (<300 nM) ATP induces currents that show little desensitization, but higher concentrations result in currents in which a rapidly inactivating component is followed by a sustained plateau. It is unlikely that the initial peak results from homomeric P2X₁ receptors also present, because it does not decline with repeated applications. It is also unlikely that the sustained component results from homomeric P2X₅ receptors, because the currents are considerably larger than those seen with P2X₅ subunits expressed alone. Thus the heteromeric receptor currents have a kinetic profile quite distinct from that observed with other homomeric or heteromeric receptors. The simplest explanation for this behavior is a prominent desensitized state that can be entered and exited only from an open state.

H. Homomeric P2X₆ Receptors

The rat P2X₆ receptor was cloned from superior cervical ganglion cDNA (81) and from rat brain (431). The human equivalent was isolated from peripheral lymphocytes as a p53 inducible gene (471). This was originally designated P2XM to reflect its abundance in human skeletal muscle (471). The mouse gene is also heavily expressed in skeletal muscle (337). The P2X₆ receptor appears to be a "silent" subunit, in the sense that no currents are evoked by ATP when it is expressed in oocytes (243, 269, 431) or HEK293 cells (431).

In the original experiments of Collo et al. (81) it was found that rat P2X₆ receptor could be expressed in HEK293 cells, but in only a tiny fraction of transfections (Fig. 2F of Ref. 81). The properties of the expressed current resembled those of the P2X₄ receptor, and this raises the possibility that these responses resulted from activation of P2X₄ receptors native to the HEK293 cells. A large fragment of the human P2X₄ receptor has been cloned from HEK293 cells (Genbank AF012903), and recently they have been shown by Northern and Western blotting to express P2X₄ RNA and protein (514). Although no one has ever reported P2X-like responses from non-transfected HEK293 cells, the possibility exists that these cells might express homomeric P2X₄ channels under certain culture conditions. A more likely explanation might be that the receptor is not sufficiently glycosylated in heterologous expression systems, given that consensus sites do not fully correspond among the various subtypes.

I. Heteromeric P2X_{2/6} Receptors

P2X₂ and P2X₆ receptors have been found to coimmunoprecipitate after expression in HEK293 cells (462). Oocytes expressing this combination have subtly differ-

ent responses to ATP than oocytes expressing only P2X₂ receptors (243). The most convincing of these differences is the fact that (at pH 6.5) the inhibition of the current by suramin is clearly biphasic; one component has the high sensitivity of homomeric P2X₂ receptors (IC₅₀ ~80 nM) (244), whereas the other component is less sensitive (IC₅₀ ~2 μM) (243).

J. Heteromeric P2X_{4/6} Receptors

Two groups have reported that P2X₄ and P2X₆ receptors form a heteromeric channel when coexpressed in oocytes (234, 269). The subunits can be coimmunoprecipitated from oocytes (269) and HEK293 cells (462). The principal functional evidence for coexpression is that currents elicited by ATP are larger in oocytes 5 days after injection of mRNAs for P2X₄ and P2X₆ than after injection of P2X₄ alone (269). However, the phenotype of the heteromer differs only in minor respects from that of P2X₄ homomers. For example, in oocytes expressing the P2X_{4/6} receptor, αβmeATP evoked a maximal current that was ~12% that caused by ATP, whereas for P2X₄ homomers this fraction was ~7% (269); the threshold concentration at which αβmeATP evoked currents is also slightly less (10 μM) in oocytes injected with both RNAs than in oocytes injected only with P2X₄ RNA (234). These small phenotypic differences highlight the difficulty in studying the properties of the heteromeric channels in an expression system in which one or both sets of homomers are also likely to be present.

K. Homomeric P2X₇ Receptors: Membrane Currents

A chimeric cDNA encoding the rat P2X₇ receptor was first constructed from overlapping fragments isolated from superior cervical ganglion and medial habenula; full-length cDNAs were subsequently isolated from a rat brain cDNA library (446). Human (380) and mouse (64) cDNAs were cloned from monocyte and microglial cells, respectively. Expression of the rat P2X₇ cDNA in HEK293 cells resulted in sensitivity to ATP as measured by inward currents (446). In the original and subsequent studies, other end points have been used, including uptake of YO-PRO-1 or similar fluorescent dyes which bind to nucleic acid and structural changes in the cell such as membrane blebbing (see sect. IVL).

1. Agonists

Four main features distinguish the currents at P2X₇ receptors from those observed at other P2X receptors. These are 1) the requirement to use concentrations of ATP greater than 100 μM, 2) the finding that 2',3'-(ben-

zoyl-4-benzoyl)-ATP (BzATP) is some 10–30 times more potent than ATP, 3) the fact that the effect of ATP (and BzATP) is much potentiated by reducing the concentration of extracellular calcium or magnesium (446), and 4) the observation that the currents can exhibit striking changes in their time course and amplitude with repeated applications of the same agonist. The first point is one of the striking similarities between heterologously expressed P2X₇ receptors and the responses of mast cells (79, 452). The second point, that BzATP is more potent than ATP, has led to the widespread use of BzATP as an agonist at P2X₇ receptors. It has also led to the erroneous belief that BzATP is selective for P2X₇ receptors; it is an effective agonist at similar or lower concentrations at other P2X receptors (25, 121). The potentiation of the responses to ATP (or MgATP) by reducing the concentration of divalent cations is a hallmark of P2X₇ responses, but a similar though smaller effect is observed with other (e.g., P2X₂) receptors. The interpretation has often been made that this indicates that ATP⁴⁻ must be the active ligand that binds to the receptor, but there is no direct evidence for this; an equally likely explanation is that the divalent ions simply bind elsewhere on the receptor and exert an allosteric inhibition, as do copper and nickel for example (see Ref. 479).

The fourth point refers to the observation that the time course of the offset of inward current evoked by ATP (at the rat P2X₇ receptor) becomes slower with successive ATP applications, and this behavior is most strikingly observed in low extracellular divalent ion concentrations (446). In the *Xenopus* oocyte expression system, the onset and offset kinetics of ATP at the human P2X₇ receptor show two components (248, 250). This suggests that under these conditions (divalent-free solutions) ATP binds to at least two sites that differ in affinity by ~50-fold. There are species differences; the human P2X₇ shows this prolongation to a lesser degree, and with the mouse P2X₇ receptor successive applications led rather to an increase in the peak amplitude of the inward current rather than a prolongation of the current (64, 183, 202, 380). The mechanism of these kinetic changes is not well understood. In the case of the mouse, rat, and human receptors, repeated brief applications of agonist (BzATP) result in a progressive increase in agonist potency so long as the initial concentration is submaximal (183).

ADP and AMP are very weak agonists at the P2X₇ receptor. However, after a brief exposure to ATP, the effectiveness of ADP and AMP is increased (although they remain weak compared with ATP) (57). A similar effect is seen on mouse microglial cells. Moreover, in the microglia, the effect translates to release of interleukin (IL)-1 β ; ADP and AMP do not normally elicit any IL-1 β release, but they do so after an initial "priming" application of ATP (57). This surprising observation suggests that a brief initial application of ATP causes a longer lasting change in

the receptor, which subsequently alters its ability to discriminate among ATP, ADP, and AMP.

One such long-lasting change might be phosphorylation. Kim et al. (238) have recently shown that the P2X₇ receptor becomes dephosphorylated on Tyr-343 as a result of exposure to agonist. When supramaximal concentrations of BzATP are applied to the rat receptor expressed in HEK293 cells, the currents show a progressive decline in amplitude; this is due to dephosphorylation of the receptor itself and can be completely prevented by phosphatase inhibitors (238). The direct demonstration that the P2X₇ receptor complex in HEK293 cells contains a receptor protein tyrosine phosphatase (RPTP β) favors the interpretation that this is activated when ATP binds to the receptor. When RPTP β dephosphorylates the receptor on Tyr-343, the current amplitude declines. This could indicate a direct effect on channel conformation (or even permeation) of the -OH group as distinct from the O-PO³⁻ group, or it could result from the disruption of a protein-protein interaction that requires the phosphotyrosine.

2. Antagonists/blockers

There are five main types of blockers. The first class is the ions. Calcium, magnesium, zinc, copper, and protons all inhibit ATP-evoked currents at the rat P2X₇ receptor; the corresponding IC₅₀ values are as follows (in μ M): 2,900, 500, 11, 0.5, and 0.4 (i.e., pH 6.1). The block is voltage independent (479). The inhibition by zinc and copper set the P2X₇ receptor apart from the other members of the family, where currents are facilitated by similar concentrations. Second, there are generic P2X receptor antagonists. Currents are relatively insensitive to block by suramin (IC₅₀ >300 μ M at rat P2X₇) and PPADS (IC₅₀ ~50 μ M) (446); the suramin analog NF279 is more potent (IC₅₀ ~10 μ M) (249). The human P2X₇ receptor appears to be more sensitive to PPADS (IC₅₀ ~3 μ M with 3-min preincubation; zero magnesium, 0.5 mM calcium) (307). The most useful blocker in this class seems to be Brilliant Blue G (215), which blocks rat P2X₇ receptors at 10 nM and human P2X₇ receptors at 200 nM. Rat P2X₂ and human P2X₄ are blocked only in the micromolar range, and others (rP2X₄, rP2X₁, hP2X₁, rP2X₃, hP2X₃, rP2X_{2/3}, and rP2X_{1/5}) are unaffected even by >10 μ M (215). Finally, oxidized ATP (ATP with the 2'- and 3'-hydroxyl moieties oxidized to aldehydes by periodate treatment) irreversibly blocks the currents when 1- to 2-h preincubation is used (446); similar concentrations (100 μ M) also block currents at P2X₁ and P2X₂ receptors (121).

The third group of blockers contains two large organic cations, calmidazolium and KN-62 (Fig. 4). Calmidazolium (10 nM) blocks currents at rat P2X₇ receptors, but not currents at cells expressing rat P2X₂ or rat P2X_{2/3} receptors (479). It is rather less effective at human P2X₇ receptors (307). This block is readily reversible and volt-

age independent; calmidazolium blocks several other ion channels, including cyclic nucleotide-gated channels, although those effects require higher concentrations (251). Calmidazolium [1-[bis(4-chlorophenyl)methyl]-3-[2-(2,4-dichlorophenyl)-2-(2,4-dichlorophenylmethoxy)-ethyl]-1H-imidazolium] has a charged imidazolium nucleus surrounded by four chlorobenzene moieties (Fig. 4) and was introduced as a calmodulin antagonist. KN-62 is a piperazine {4-[2-[(5-isoquinolinesulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl]phenyl ester} (Fig. 4) used as an inhibitor of calcium/calmodulin-dependent protein kinase type II (CaM kinase II). It blocks currents in cells expressing the human P2X₇ receptor but has little effect at the rat P2X₇ (202). Neither of these actions appears to be related to calmodulin or CaM kinase II.

The studies with blockers are difficult to compare, even for the same species. The IC₅₀ values are quite approximate because of their dependence on the agonist concentration; where possible, the values quoted correspond to inhibition of the response elicited by a just-maximal agonist concentration. The time of preincubation of blockers such as PPADS greatly affects the potency but varies from study to study. Some experiments are carried out in normal physiological solution (2 mM calcium, 1 mM magnesium), and others are not.

The fourth class of P2X₇ antagonist described is 17 β -estradiol. Cario-Toumaniantz et al. (55) reported block of currents activated by BzATP in COS cells expressing the human P2X₇ receptor (and also a human macrophage line U-937). This effect did not involve genomic estrogen receptors: the EC₅₀ was ~ 3 μ M, and progesterone and 17 α -estradiol were essentially without effect. Finally, receptor blockade by a monoclonal antibody has also been reported; this is selective for human P2X₇ receptors (43). A monoclonal antibody raised against the rat receptor potentiates rather than inhibits the currents at rat P2X₇ receptors (238).

3. Permeation properties

Currents through the P2X₇ receptor show little or no rectification. With brief agonist applications, the channel has low permeability to NMDG, but this increases as the agonist application is prolonged (446, 480). The time constant for the increase in permeability (P_X/P_{Na}) increases from ~ 1 s (X = dimethylamine) to 4 s (X = Tris) to 10 s (X = NMDG). The time constants are similar to those observed in those cells expressing P2X₂ receptors that show an increase in NMDG permeability; however, the increase in permeability is seen in all transfected HEK293 cells rather than a proportion of them as P2X₂ and P2X₄ receptors (see sects. *ivB3* and *ivD3*). Even when NMDG is permeable, the pore remains cation selective (480). The concentrations of BzATP that are required to open the channel initially are the same as those which cause dila-

tion; the rate of dilation increases steeply from 0.3 to 30 μ M BzATP (480). The permeability measurements are carried out in bi-ionic conditions, without extracellular calcium or magnesium. The addition of these divalents (1 mM magnesium, 2 mM calcium) slows the rate of increase in permeability to NMDG but does not change the final value (481).

There have been attempts to observe heteromeric channels. According to the biochemical experiments of Egan, Voigt and associates (462), P2X₇ receptors do not coimmunoprecipitate with other receptors (see Table 4). When P2X₁ and P2X₇ receptors are coexpressed in HEK293 cells, the currents elicited by BzATP resemble those expected from a mixture of two independent sets of homomeric channels (56).

4. Desensitization/inactivation

In HEK293 cells, the inward current evoked by ATP or BzATP shows no desensitization during applications lasting for many seconds (Fig. 5). Longer applications result in the increase in permeability described above, and this is sometimes accompanied by an increase in the current amplitude.

L. Homomeric P2X₇ Receptors: Other Measures of Activation

1. Uptake of calcium and fluorescent dyes

The commonly used dyes (ethidium and YO-PRO-1) are shown in Figure 8. They become fluorescent when they intercalate nucleic acids, and this therefore gives a direct measure of their entry into cells. They have the advantage that they can be added in relatively low concentrations (typically ~ 1 μ M) to an otherwise physiological solution. There is no easy way to correlate the intensity of the fluorescence signal with the concentration of dye in the cell; however, by taking the first time derivative of the fluorescence intensity it is possible to estimate the rate of entry of dye (480). Such experiments show that the time course of YO-PRO-1 (several seconds) is considerably slower than the ionic current in normal conditions (several tens of milliseconds); it is, however, comparable to the time course of inward current when NMDG is used as the extracellular cation (480). This appears to be true for expression in either HEK293 cells (446, 480) or COS cells (56). This is consistent with the interpretation that NMDG and cationic dyes such as YO-PRO-1 share a common permeation pathway. In most other respects, the properties of ATP-evoked YO-PRO-1 uptake closely resemble those of ATP-evoked ionic current: with brief applications both are fully reversible, the effective concentrations of ATP and BzATP are similar, the sensitivity to block by magnesium (446) and other ions is similar

(479), and the degree of block by other agents generally corresponds. The most notable exception here is calmidazolium, which blocks the ionic current (see above) but not the uptake of YO-PRO-1 (479). There are clear species differences among the P2X₇ receptors when dye uptake is measured, and these correlate with the differences seen when measuring ionic current (183, 380). There are also species differences in the potency of the block by isoquinolines such as KN-62, with human and mouse receptors being more sensitive than rat receptors; this applies whether YO-PRO-1 uptake or ionic current is measured (202).

For heterologously expressed P2X₇ receptors, the progressive increase in permeability to NMDG has been observed in HEK293 cells (rat P2X₇, Refs. 446, 480, 481; human P2X₇, Ref. 380; mouse P2X₇, Ref. 64) and oocytes (rat P2X₇, Ref. 229). The uptake in YO-PRO-1 has been shown in HEK293 cells (rat P2X₇, Refs. 446, 480, 481; human P2X₇, Ref. 380), COS cells (human P2X₇, Ref. 56; ethidium uptake), and oocytes (rat P2X₇, Ref. 229; *Xenopus* P2X₇, Ref. 363). However, two groups have sought the permeability change in oocytes and failed to observe it (rat P2X₇, Ref. 367; human P2X₇, Ref. 248). This suggests that the host cells might contribute critical molecules that are required for the pore dilatation to occur, and this possibility is discussed further below.

Bianchi et al. (25) measured the uptake of calcium into I321 astrocytoma cells transfected to express human P2X₇ receptors; this peaked at ~10 s after adding BzATP (25 μ M). This calcium signal was blocked by <10 μ M PPADS (3-min preincubation). Dubyak and colleagues (401, 402) observed the entry both of calcium and fluorescent dyes in transfected HEK293 cells. Both intracellular Ca²⁺ ([Ca²⁺]_i) and ethidium fluorescence rose within a few seconds of applying BzATP. Maitotoxin produced similar effects, but these were of slower time course and were observed in both HEK293 cells either transfected or not with the P2X₇ cDNA. The entry pathway for the dyes was similar whether activated through P2X₇ receptors or maitotoxin receptors, in the sense that ethidium entered more readily than YO-PRO-1, and POPO₃ hardly entered at all. The experiments disprove the hypothesis that maitotoxin directly activates P2X₇ receptors but leave open the possibility that a common entry pathway for the fluorescent dyes is activated through two distinct receptors.

There has been little by way of systematic structure-function analysis of the P2X₇ receptor. Truncation of the protein (deletion of residues from 419 to 595) results in a receptor with much reduced uptake of YO-PRO-1 (446). Human P2X₇ receptors with the point mutation E496A occur as a result of a single nucleotide polymorphism; when expressed in HEK293 cells, these receptors show a reduced uptake of ethidium in response to ATP (164). This residue is at the center of a highly conserved charged

motif in the COOH-terminal tail (His-Arg-Cys-Leu-Glu-Glu-Leu-Cys-Cys-Arg-Lys-Lys) (Fig. 1). The recognition of domains involved in protein-protein interactions in the COOH terminus of the P2X₇ receptor should prompt further studies by mutagenesis. These include binding sites for bacterial lipopolysaccharide (94), an SH₃ domain (94, 238), and a region similar to sequences known to bind α -actinin (238).

2. Membrane blebbing and morphological changes

ATP or BzATP induces remarkable changes in the appearance of HEK293 cells transfected with the rat P2X₇ receptor (294, 480). After ~30 s of continuous application of BzATP (30 μ M), the plasma membrane begins to develop large blebs, and after 1 or 2 min, these become multiple and sometimes coalesce. The time to the appearance of the first bleb can be delayed by removal of extracellular sodium or, in cases when patch-clamp recording is being made, by using sodium as the principal intracellular cation. Membrane blebs develop as large, hemispherical protrusions of plasma membrane, ranging in diameter from 1 to >10 μ m. They are usually preceded by the appearance of smaller vesicles (<1 μ m diameter), which often become very numerous and are shed from the cell (294).

Taken together, it would appear that several distinct sequelae can now be ascribed to activation of the homomeric P2X₇ receptor. The earliest event has been studied electrophysiologically, usually with agonist applications up to several seconds. This is the opening of a cation-selective ion channel; it can occur within milliseconds (with a maximal agonist concentration). If the agonist application is repeated, the current induced becomes larger and takes longer to decline after each application, but here there are species differences. If the agonist application is prolonged (several seconds), there is an increase in permeability to larger organic cations, including NMDG (measured as ionic current) and YO-PRO-1 (measured by cell fluorescence). A key question that is raised is whether these two properties are intrinsic to the P2X₇ receptor protein or whether they require additional molecules to be provided by the host cell (Fig. 10).

The simplest explanation is that both these properties are intrinsic to the P2X₇ receptor protein (Fig. 10A). In favor of this interpretation are the following observations. 1) The increase in permeability is progressive; it occurs more quickly for smaller cations such as dimethylammonium and TEA and more slowly for larger cations such as NMDG and YO-PRO-1. 2) It is observed in a range of host cells (HEK293, COS, and oocytes). 3) Several procedures that block the initial current also block YO-PRO-1 uptake. These include Brilliant Blue G and polyethylene glycols (480). 4) The two properties are shown not only by P2X₇ receptors, but also in a proportion of

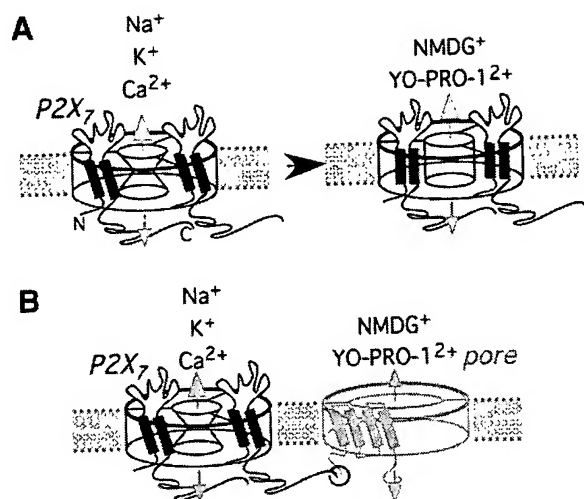


FIG. 10. Schematic illustration of two contrasting mechanisms for the time-dependent increase in permeability observed for P2X₇ receptors. *A*: pore dilatation. Several subunits (two indicated) form a channel permeable to small cations. This opens within milliseconds after binding ATP but undergoes a conformational change (arrow) that is associated with dilatation of the ion conducting pathway. *B*: activation of a distinct channel protein. The P2X₇ receptor (*left*) forms a channel permeable to small cations, as in *A*. The activated receptor interacts with (directly, as indicated by circle, or through further intermediate proteins) and opens a distinct channel protein (shown in gray), which is permeable to larger cations including fluorescent dyes. Evidence for and against these models is discussed in the text.

cells expressing P2X₂, P2X_{2/3}, and P2X₄ receptors, and the kinetics are similar in each case. For those receptors, point mutations in the second membrane-spanning domain can alter the NMDG permeability increase (see sects. *1vB3* and *1vE3*).

On the other hand, one might postulate that the P2X receptors simply activate an intrinsic (yet unidentified) membrane protein that functions as a permeation pathway for large cations and YO-PRO-1. Several results are more easily reconciled with this interpretation. 1) Calmidazolium blocks the current while leaving the YO-PRO uptake unaffected. 2) Maitotoxin can activate a dye entry pathway with very similar properties to that seen with P2X₇ receptors (although slower kinetics), and the P2X₇ receptor is not required for this (401, 402). 3) In some oocyte expression systems, activation of the P2X₇ receptor results only in the first response (opening a channel permeable to small cations) and not the second (there is no NMDG permeability, or YO-PRO-1 uptake) (367; human P2X₇, Ref. 248). Similarly, YO-PRO-1 uptake varies considerably among different transfections of HEK293 cells, even though ionic currents are comparable (unpublished observations).

The plasma membrane blebbing and microvesiculation that occurs on activation of P2X₇ receptors has not been seen for other members of the family and seems likely to reflect the engagement of downstream signaling

mechanisms that are unrelated to the movement of ions across the membrane. It would be useful to engineer point mutations that can selectively prevent the flow of ionic current and others that inhibit the membrane bleb and vesicle formation.

A final end point of P2X₇ receptor activation is indisputably cell death. The literature is confused here, because of the many different ways in which death has been defined. For example, experimenters with fluorescent-activated cell sorters sometimes use YO-PRO-1 uptake to identify dead cells; cells expressing P2X₇ receptors can take up YO-PRO-1 repeatedly, and electrophysiological recordings indicate at such a time that they are far from dead (480). The release of lactic dehydrogenase activity into the medium is sometimes used as a measure of cell death; this occurs only after many tens of minutes of continuous application of BzATP to HEK293 cells transfected with P2X₇ receptors (294, 480).

V. P2X RECEPTORS IN NATIVE CELLS AND TISSUES

A. Brain Neurons

Norenberg and Illes (343) have provided a fairly comprehensive account of studies on central P2X receptors; further brief reviews are by Khakh (235) and Robertson et al. (384).

1. Exogenous ATP

The effects of exogenous ATP have been studied by intracellular and/or whole cell recordings made from neurons in slices of hippocampus (355–357), supraoptic nucleus (414), motor nucleus of the Vth nerve (84, 230), mesencephalic nucleus of the Vth nerve (232), locus ceruleus (342, 413), medial habenula (106, 107, 383), hypoglossal nucleus (141), and nucleus tractus solitarius (226), as well as in dissociated cells in the case of hippocampus (357), supraoptic nucleus (414), tuberomammillary nucleus (142), dorsal motor nucleus of vagus (317), mesencephalic nucleus of Vth nerve (84), and nucleus of the solitary tract (317, 468). Four main effects have been described.

A) INWARD CURRENT. The current evoked by ATP usually (84, 232, 317, 414, 468) but not always (142) shows prominent inward rectification. Only in a few cases have any further properties of the permeation pathway been described; in histaminergic tuberomammillary neurons (142) and in neurons of the nucleus of the solitary tract (468), P_{Ca}/P_{Na} was ~ 1.2 (at 2 mM extracellular $[Ca^{2+}]$). This relatively low value is similar to that of the heteromeric P2X_{2/3} receptor, and considerably lower than that seen for the homomeric P2X₂ or P2X₄ receptors. Only in

a few cases have the pharmacological properties of the currents been investigated thoroughly. In general, this is more reliable with dissociated cells, where problems of nucleotide degradation are reduced. In dissociated cells identified as vagal motoneurons, the EC_{50} for ATP is ~ 50 μ M, and $\alpha\beta$ meATP has no effect at 100 μ M; suramin inhibits currents elicited by ATP (50 μ M) with an IC_{50} of 10 μ M (317). In cells dissociated from the mesencephalic nucleus of Vth nerve (proprioceptive and mechanosensitive primary afferent cell bodies), ATP elicits inward currents (EC_{50} 3 μ M), but $\alpha\beta$ meATP does not (232).

In summary, inward currents in response to exogenous ATP are readily observed in neurons dissociated from several regions of the mammalian brain. On the other hand, responses to exogenous ATP that can be attributed to P2X receptor activation are difficult to observe in brain neurons in slices, and generally require very much higher agonist concentrations. It seems probable that the high levels of ATP released from damaged cells during the preparation of the slice, and perhaps also during the continued incubation of the slice in vitro, desensitizes (or internalizes) P2X receptors. It is also possible that intimate interactions between the ectodomain of the receptor and the extracellular matrix proteins present access barriers that are disrupted by cell dissociation.

B) PRESYNAPTIC ACTION. The second effect that has been reported in intact slices of brain tissue is a presynaptic stimulation of the release of glutamate (206), best evidenced as the increase in frequency of spontaneous synaptic currents (226, 230, 231). Neurons of the motor nucleus of the trigeminal (Vth) nerve receive a prominent excitatory input from primary afferents that have their cell bodies in the mesencephalic nucleus. ATP elicits spontaneous glutamate-mediated excitatory postsynaptic potentials (EPSCs) in the motor neurons; the receptor involved has not been characterized pharmacologically in any detail but differs in its rate of desensitization from that on soma of the same cells in the mesencephalic nucleus (84, 231). In slices of motor nucleus of Vth nerve (230), the increase in EPSCs was blocked by cadmium, implicating depolarization of nerve terminals and activation of voltage-gated calcium channels, whereas in slices of nucleus tractus solitarius (226), sufficient calcium enters through the P2X receptors themselves to bring about the increased transmitter release. The effects of endogenous ATP and congeners in the brain stem are of particular interest with respect to in vivo studies. Spyer and colleagues (377) have shown that unilateral microinjection of ATP or $\alpha\beta$ meATP into the ventrolateral medulla excites neurons and reduces resting phrenic nerve discharge (an indication of central inspiratory drive) (457). Identified inspiratory neurons in the pre-Botzinger complex are excited by $\alpha\beta$ meATP and CO_2 (458), and this has led to the suggestion that the effects of acidosis might

result from potentiation of the effects of endogenous ATP at P2X₂ receptors (436).

C) INCREASE IN $[Ca^{2+}]_i$. Responses of dissociated neurons have also been recorded by imaging changes in intracellular calcium (hippocampus, Ref. 357; hypothalamus, Ref. 62; cerebellar Purkinje cells, Ref. 301; rat supraoptic neurons, Ref. 414; neurohypophysis, Ref. 465). In the case of the Purkinje cells, the response to ATP is not mimicked by $\alpha\beta$ meATP, is potentiated by acidification and by zinc, and is blocked by suramin (IC_{50} 50 μ M) and PPADS (IC_{50} 6 μ M), although not by Ip5I; these results indicate that P2X₂ receptor subunits dominate the pharmacological properties of the calcium entry pathway (148). In the hippocampus, the $[Ca^{2+}]_i$ signal is mimicked by $\alpha\beta$ meATP, reduced by PPADS, and only little affected by thapsigargin (357).

ATP elicits the release of arginine vasopressin, although not oxytocin, from posterior pituitary terminals; the EC_{50} is ~ 9 μ M (465). An increase in $[Ca^{2+}]_i$ is observed in a subset of these neurohypophysial terminals when ATP (EC_{50} 5 μ M) is applied; the response required extracellular calcium but was unaffected by blockers of voltage-gated calcium channels. $\alpha\beta$ MeATP (100 μ M) had much less effect than ATP, and the action of ATP was reversibly abolished by suramin (300 μ M). Pubill et al. (373) have suggested that disaggregation of actin consequent to calcium entry may also play a role. It was proposed that ATP might have a local paracrine action to enhance the release of arginine vasopressin at the level of the neurohypophysis and that the receptor involved most closely resembled the P2X₂ receptor.

D) SINGLE-CHANNEL OPENING. The fourth type of response to exogenous ATP is the stimulation of single-channel openings in membrane patches from rat hippocampal granule cells (509). In 19 of 98 outside-out patches, ATP elicited openings of a 56-pS channel. The unitary current showed a linear voltage dependence and was unaffected by changes in calcium from 0.3 to 0.85 mM. $\alpha\beta$ MeATP (40 μ M) opened similar channels also in a small proportion (3 of 17) of patches. The maximal overall probability of the channel being open (p_o , with 1 mM ATP) was about 0.1, but openings occurred in obvious bursts within which p_o was much higher (0.96). Suramin (40 μ M) reduced the probability of opening by reducing the mean open time and the mean burst length, a result not consistent with simple competitive antagonism. In some patches, suramin increased the unitary currents; this finding is of interest because suramin has been reported to increase currents elicited by ATP in myenteric neurons (12) and in oocytes expressing homomeric P2X₄ receptors (29). Outside-out patches from hypothalamic paraventricular neurons also show predominantly flickery channel openings (498). As the authors point out, the properties of these ATP-activated channels in dentate granule cells and hypothalamic cells do not correspond to those of any of the combina-

tions of subunits so far studied by heterologous expression.

2. Endogenous ATP

Postsynaptic currents mediated by release of endogenous ATP have been described for the hippocampus (CA1, Refs. 355–357; CA3, Ref. 313), medial habenula (106), and locus ceruleus (342). The main evidence for this conclusion is the finding that the currents are not inhibited by high concentrations of antagonists at AMPA/kainate, NMDA, serotonin (5-HT₃), or nicotinic acetylcholine receptors, whereas they are depressed by suramin or PPADS. In CA1 cells and medial habenula, the synaptic currents and the currents elicited by exogenous ATP show relatively little inward rectification (107, 356). In the case of the hippocampus, the synaptic currents are potentiated by zinc (10 μ M), consistent with the involvement of a P2X₂ or P2X₄ subunit. There is evidence that distinct presynaptic fibers release ATP and glutamate in the medial habenula, because release of glutamate (but not ATP) is selectively inhibited by adenosine acting at presynaptic A₁ receptors (383). The amplitudes of the ATP-mediated synaptic currents recorded are uniformly small (typically 20–50 pA) compared with EPSCs mediated by excitatory amino acids (typically >1 nA), and this certainly raises questions regarding the physiological circumstances under which such synaptic transmission comes into play. It is possible that the currents are small because ATP released from dying cells results in continued receptor desensitization. Alternatively, small-amplitude currents might have significant signaling consequences quite distinct from those of the depolarization, such as calcium-mediated cytoskeletal changes that contribute to synaptic remodeling.

There are difficulties in pursuing the physiological role for P2X receptors activated by endogenous ATP. The first remains the inadequacy of the antagonists available. It must be stressed that, at the concentrations used in many experiments (>30 μ M), suramin, PPADS, and reactive blue 2 have been shown to block currents elicited by kainate, NMDA, and GABA in dissociated cells (328) and to slow the rate of rise of currents elicited by AMPA (165). A second complication is that evoked synaptic currents are often observed in only a fraction of neurons tested, and this may make it difficult to carry out the critical comparative studies in tissues from mice in which P2X receptor subunits have been knocked out. A third difficulty arises from the pronounced desensitization that is often observed when ATP and related nucleotides are applied to brain neurons. In the experiments on the CA1 pyramidal cells (356), the purinergic component of the EPSC declined to zero when it was elicited at stimulation frequencies >0.06 Hz. It may be possible to address the

problem of desensitization or internalization of receptors due to tonically high ambient extracellular ATP levels by adding ATP-degrading enzymes to the *in vitro* solution.

A recent ultrastructural study localized P2X₄ and P2X₆ subunits to the peripheral regions of the postsynaptic density in hippocampal and Purkinje neurons (392), and attention has now been drawn to a possible role in modulating glutamate-mediated synaptic transmission. Recording the extracellular field excitatory postsynaptic potential, Pankratov et al. (357) found that a 200-ms train of stimuli at 200 Hz was insufficient to elicit long-term potentiation (LTP) at CA1 synapses; a train 1 s in duration evoked robust LTP. However, in the presence of PPADS (20 μ M), even the shorter train evoked LTP. Using intracellular recordings, they showed that the NMDA component of the CA1 EPSC was inhibited during continuous stimulation of the Schaffer collaterals; this has been ascribed to a rise in intracellular calcium inhibiting the postsynaptic response of the NMDA receptor (see Ref. 389). This inhibition of the NMDA component of the EPSC was also blocked by PPADS (20 μ M), leading Pankratov et al. (357) to reason that calcium entry through postsynaptic P2X receptors may be reducing the NMDA component. In isolated cells, they showed directly that application of ATP (or $\alpha\beta$ meATP) significantly inhibited the current evoked by exogenous NMDA. This inhibition was not seen when barium replaced calcium in the superfusing solution, implying that it resulted from calcium entry through P2X receptors.

B. Retina

P2X receptor mRNAs have been detected in several retinal cell types (38, 39, 209, 496), but the principal functional studies have been carried out on ganglion cells (sect. vF5), Muller cells (sect. vD1), and pigment epithelial cells (sect. vG10).

C. Spinal Cord Neurons

1. Exogenous ATP

Exogenous ATP elicits inward currents in dorsal horn neurons in slices (13) or cells cultured from neonates (165, 166, 199, 211). The currents show marked inward rectification (13, 199). The action of ATP is not mimicked by $\alpha\beta$ meATP (199). The increase in $[Ca^{2+}]_i$ produced by ATP in acutely dissociated dorsal horn neurons probably reflects entry through P2X receptors, because it is not affected by enough lanthanum (30 μ M) to block the $[Ca^{2+}]_i$ elevation elicited by high potassium concentrations (13); this effect is not mimicked by $\alpha\beta$ meATP (100 μ M), but the action of ATP (100 μ M) is completely blocked by suramin (100 μ M).

ATP elicits the release of glutamate, GABA, and glycine in the spinal cord. Gu and MacDermott (166) showed that ATP (and $\alpha\beta\text{meATP}$) increased the frequency of spontaneous glutamate-mediated EPSCs in embryonic rat dorsal horn cells cocultured with sensory neurons from the dorsal root ganglia. The increase in frequency persisted in tetrodotoxin but required extracellular calcium; experiments with lanthanum indicated that most of the calcium entered through P2X receptors themselves as distinct from voltage-dependent calcium channels opened by the ATP-induced depolarization. By focal application, it was shown that the P2X receptors were on neurites arising from dorsal root ganglion cells, as they made contacts with the dendrites of spinal cord neurons. Also in intact slices from rat spinal cord, the excitation of preganglionic sympathetic neurons by BzATP was prevented by glutamate receptor antagonists (95). This action was inhibited by Brilliant Blue G, suggesting that it resulted from activation of P2X₇ receptors on glutamate-containing presynaptic terminals.

More recent studies on spinal cord slices have provided key information regarding the further identification of the presynaptic fibers in the dorsal horn from which glutamate release is increased (321, 322). Spontaneous release of glutamate from terminals synapsing onto lamina V cells was much increased by $\alpha\beta\text{meATP}$ and by capsaicin. However, in the presence of tetrodotoxin to block signaling between neurons in the cord, the action of $\alpha\beta\text{meATP}$ persisted whereas the effect of capsaicin was blocked. This synaptic input to lamina V cells from $\alpha\beta\text{meATP}$ -sensitive, capsaicin-insensitive fibers originates from primary afferent inputs of the A δ class (322); these probably correspond to the $\alpha\beta\text{meATP}$ -sensitive (P2X_{2/3} receptor-expressing) A δ fibers responsible for mechanical allodynia (467) (Fig. 11). This would be consistent with behavioral studies reporting a reduction in the mechanical allodynia following spinal nerve ligation in rats treated intrathecally with antisense oligos directed against the P2X₃ subunit (192) and confirms the suggestion by Ossipov et al. (351) that mechanical allodynia involves capsaicin-insensitive A fibers. Lamina II neurons, on the other hand, receive glutamate EPSCs from $\alpha\beta\text{meATP}$ -sensitive terminals that are also sensitive to capsaicin (322). These presumably originate from the P2X₃/VR1-expressing subset of small/medium-sized dorsal root ganglia (see sect. *vF2*) and which contribute to the nociceptive behavior elicited by Formalin; this is reduced in P2X₃ knock-out mice (76, 433) or in mice treated with P2X₃ antisense oligonucleotides (192).

GABAergic spontaneous miniature inhibitory postsynaptic (IPSCs) are also increased in frequency by ATP (in 22% of synapses studied), although not by $\alpha\beta\text{meATP}$ (199) (Fig. 11). This effect also requires entry of extracellular calcium, at least partly through the P2X receptors themselves. These experiments were carried out on cul-

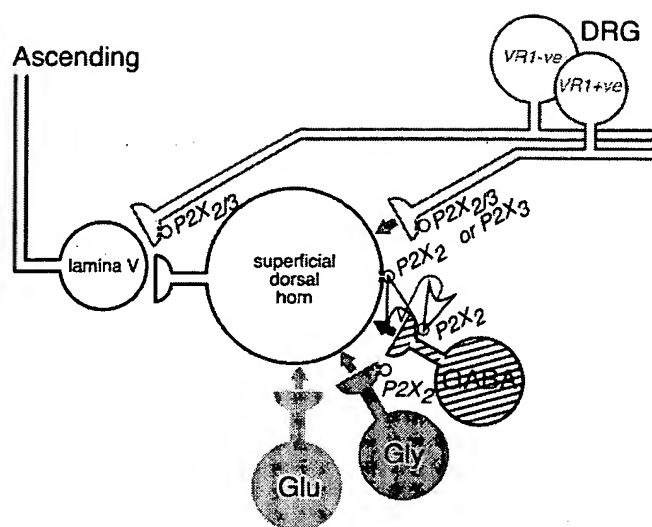


FIG. 11. Schematic indication of the actions of ATP at P2X receptors on neurons in the dorsal horn of the spinal cord. Neurons in the superficial dorsal horn receive synaptic inputs from glutamate, GABA, and glycine terminals; the frequency of glutamate-mediated spontaneous excitatory postsynaptic currents (EPSCs) is increased by $\alpha\beta$ -methylene ATP (implicating P2X₃ and/or P2X_{2/3} receptors) and by capsaicin. The frequency of GABA- and glycine-mediated EPSCs is increased by ATP but not by $\alpha\beta$ -methylene ATP (implicating P2X₂-like receptors). A small fraction of neurons receives an ATP-mediated synaptic input. GABA-releasing neurons also release ATP. Neurons in deeper dorsal horn (lamina V) receive synaptic inputs from glutamate-releasing terminals that are activated by $\alpha\beta$ -methylene ATP though not by capsaicin. Capsaicin can release glutamate onto deeper neurons, but this is due to excitation (tetrodotoxin-dependent) of more superficial interneurons. VR1+ve and VR1-ve indicate sensory neurons that either express or do not express vanilloid receptor type 1, respectively. [Data from Jo and Schlichter (219), Nakatsuka and Gu (322), Nakatsuka et al. (321), Gu and MacDermott (166), Hugel and Schlichter (199), and Rhee et al. (382).]

tures of dorsal spinal cord, from 3- to 4-day-old rats, and many of the cells receiving the GABAergic inputs were themselves depolarized by the ATP. No effect of ATP was observed on spontaneous glutamate-mediated EPSCs. A rather similar effect was reported by Rhee et al. (382) for pharmacologically isolated glycine-mediated IPSCs. In this case, dorsal horn cells were acutely dissociated from 10- to 14-day-old rats so that their normal synaptic inputs remained mostly intact. In more than half the cells, ATP increased the frequency of the spontaneous miniature IPSCs, and this strong facilitatory action largely persisted in cadmium (100 μM), which blocked voltage-gated calcium channels. $\alpha\beta\text{MeATP}$ had no effect.

2. Endogenous ATP

A role for endogenous ATP has been proposed in the dorsal horn, because synaptic currents can be evoked that are sensitive to blockade by suramin and PPADS. In the work of Bardoni et al. (13), the effective concentrations of the antagonists are very high (500 μM suramin, 100 μM PPADS), and these authors recognize the difficulties in

making conclusions about the identity of the underlying transmitter (165). Jo and Schlichter (219) described an EPSC that was reversibly inhibited by suramin (30 μ M, 80% inhibition) and PPADS (50 μ M, 50% inhibition). The ATP-mediated EPSC is linearly dependent on voltage, but this is different from the properties of the current evoked by exogenous ATP. These experiments on the ATP component of the synaptic current are carried out in the presence of a cocktail of antagonists, typically bicuculline, strychnine, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 2-amino-5-phosphonopentanoic acid (AP-5), to block EPSCs mediated by GABA, glycine, AMPA/kainate, and NMDA receptors, respectively (13, 219). However, by washing out the bicuculline and separating the responses by setting the membrane potential to either the cation (for P2X receptors) or chloride (for GABA_A receptors) reversal potential, Jo and Schlichter (219) were able to show that the same stimuli that elicited ATP currents also evoked GABA currents; this suggests corelease of the two transmitters. Despite the isolation of an evoked synaptic current mediated by ATP, spontaneous synaptic currents have not been observed (either in the spinal cord or elsewhere in central neurons). The analysis of ATP-mediated spontaneously occurring synaptic currents, readily observed at the peripheral neuroeffector junction such as the vas deferens (52), would be an important step toward understanding the mechanism by which ATP is released at central synapses. Figure 11 summarizes in schematic form our present understanding of the role of P2X receptors on cells in the dorsal horn; note that the evidence is taken from several different experimental approaches.

D. Glial Cells

Müller cells are one of the principal glial cells of the retina (with astroglia and microglia). Activation of P2X receptors elicits an inward current (human, Ref. 358) and a rise in $[Ca^{2+}]_i$ (rat, Ref. 338; rabbit, Refs. 288, 358). The electrophysiological response of the human Müller cells has several features of P2X₇ receptors; BzATP (effective at 5–50 μ M) is more potent than ATP (α BmeATP had no effect), the currents show little rectification or desensitization (even over 5 min), and the currents are strongly inhibited by KN-62 (1 μ M) and by extracellular magnesium (358). On the other hand, there was no significant permeability to NMDG or uptake of fluorescent dye such as YO-PRO-1. P2X₇ mRNA was detected by RT-PCR in these human Müller cells (358), although not in rat Müller cells (209).

Several studies have described the responses of glial cells to ATP, including Schwann cells (6, 85, 167, 170, 208, 490; reviewed in Ref. 476). There is clear evidence that paracrine signaling by ATP is responsible for the spread of calcium waves among cortical astrocyte cells in culture

(85, 170), and this has been reviewed (135). Although human astrocytes can express P2X₇ receptors, most evidence indicates that the calcium waves involve receptors of the P2Y class (220), and it is not discussed further here.

E. Autonomic Neurons

The P2X₂ receptor subunit has a widespread tissue distribution in autonomic neurons, but it is generally found to be coexpressed with one or more other subunits. The distribution of the subunits in various peripheral neurons has been usefully reviewed by Dunn et al. (104).

1. Pheochromocytoma cells

Pheochromocytoma (PC12) cells have a long history as model cells for the study of ATP responses and are included here because of their resemblance to sympathetic neurons. Inoue et al. (206) found that ATP caused norepinephrine release from PC12 cells and that this appeared not to involve voltage-gated calcium channels. Nakazawa et al. (325) showed that ATP elicited a current that was cation selective, with significant permeability to TEA and Tris and very little permeability to glucosamine. They observed a significant calcium permeability (P_{Ca}/P_{Na} 5.4, corrected for ion activities) at external calcium concentration of 1.8 mM, and also observed that further increases in the calcium concentration led to a progressive block of the current. The concentration of calcium ions causing half-maximal block of the current was ~6 mM, which is close to that observed for homomeric P2X₂ receptors (482). The calcium that enters through P2X receptors can engage downstream signaling functions such as activation of mitogen-activated protein kinase (448). The ATP-induced current in PC12 cells shares other properties with the P2X₂ receptor, including potentiation by protons (245, 440, 441), voltage dependence, and block by di- and trivalent cations (329, 330).

2. Sympathetic neurons

In rat superior cervical ganglion cells, ATP evokes inward currents (73, 233, 324, 327, 386, 387) and elicits the release of norepinephrine (30, 487). Rogers and Dani (387) measured directly the calcium permeability of the P2X receptors by simultaneous measurements of intracellular calcium and membrane currents. In physiological solution (2.5 mM calcium) at –50 mV, some 6.5% of the ATP-evoked current was carried by calcium, compared with 12.4 and 4.7% for channels activated by *N*-methyl-D-aspartate and acetylcholine on the same cells. The calcium flux through the P2X receptors is sufficient to evoke the release of norepinephrine. Boehm et al. (30, 31) showed that norepinephrine was released by ATP from cultures for rat superior cervical ganglion cells, even

when voltage-gated calcium channels were blocked by cadmium (see also Ref. 487). This effect was also seen for culture of neurites, separated from their original cell bodies.

The currents show relatively slow desensitization, and the action of ATP is not mimicked by $\alpha\beta\text{meATP}$; the underlying unitary currents are ~ 14 pS (see Ref. 124). The currents are potentiated by zinc (73). This would be consistent with a receptor composition comprising P2X_2 , P2X_4 , and/or P2X_6 subunits, and immunohistochemical studies show that these three are the most abundant forms expressed on rat superior cervical ganglion cells (510).

Guinea pig sympathetic neurons have different properties from those of the rat. Evans et al. (120) and Khakh et al. (233) showed that in the celiac ganglion cells the currents evoked by ATP were mimicked by $\alpha\beta\text{meATP}$, and this is also true for most cells in the superior cervical ganglion (516). These observations are consistent with the cells expressing heteromeric $\text{P2X}_2/\text{P2X}_3$ receptors. Immunohistochemical studies with antibodies raised against the COOH terminus of the rat P2X_2 receptor revealed staining in most superior cervical ganglion cells; P2X_3 immunoreactivity was seen in a subpopulation of neurons, and immunoreactivity for P2X_1 , P2X_4 , P2X_5 , and P2X_6 receptors was not observed (516).

ATP mediates synaptic potentials in guinea pig cultured celiac neurons (120). One of the main targets of these cells in vivo is the mesenteric vasculature, and Evans and Surprenant (123) had previously shown that the excitatory junction potential recorded from that vascular smooth muscle was mediated by ATP. In culture, the cells make synapses on each other; focal stimulation of nerve cell processes within the culture evokes synaptic currents of ~ 200 pA at resting potentials. These currents are unaffected by antagonists at nicotinic, glutamate, or 5-HT_3 receptors, but they are blocked by suramin ($\text{IC}_{50} \sim 3$ μM) or by continuing an application of $\alpha\beta\text{meATP}$ until the current that it evokes has desensitized. Spontaneous synaptic currents mediated by ATP have also been reported in these neurons (416, 417).

There are also differences between guinea pig and rat in the responses of chromaffin cells dissociated from the adrenal medulla (286). ATP induces in guinea pig cells a slowly desensitizing current that is not mimicked by $\alpha\beta\text{meATP}$, more suggestive of P2X_2 than $\text{P2X}_2/\text{P2X}_3$ heteromers. On the other hand, ATP did not induce any currents in rat cells, despite the observation that they express both P2X_1 and P2X_2 immunoreactivity (488).

3. Parasympathetic neurons

Rat (134) and guinea pig (4) cardiac ganglion cells respond to ATP. The rat cells show a fast-onset, inwardly rectifying, cation-selective current that is desensitized by

$\alpha\beta\text{meATP}$ and blocked either by increasing the calcium concentration or by reactive blue 2 ($\text{IC}_{50} \sim 1$ μM). The relative permeabilities of the monovalent inorganic and organic cations were thoroughly measured in rat submandibular ganglion cells; these are similar to those of cloned rat P2X_2 receptors expressed in mammalian cells (285). The effects of protons were also similar to those seen for the cloned homomeric P2X_2 receptor. Intracellular dialysis with antibodies raised against the COOH terminal of the P2X_2 or P2X_4 (but not P2X_1) subunits reduced the currents elicited by ATP. Taken together, these results suggest that the receptor in these cells might be a heteromer including P2X_2 and P2X_4 subunits (285). These dissociated neurons also express immunoreactivity for P2X_2 and P2X_4 subunits (420). However, the intact ganglia show only P2X_5 immunoreactivity, and recordings from neurons in the intact ganglia do not respond to ATP. This observation is similar to that made by Stebbing et al. (437) for dorsal root ganglia (see sect. *vF3*) and clearly indicates that the procedures used for dissociation of cells have profound but little understood effects on the membrane expression of P2X receptor subunits. Clearly, this is an area that will repay future study.

In the guinea pig, a transient response was distinguished from slower currents; this reversed close to 0 mV, but no systematic permeability measurements were made. About 40% of rat pelvic ganglion cells show robust responses to ATP that have all the characteristics of the P2X_2 receptor, including potentiation by protons and zinc, ineffectiveness of $\alpha\beta\text{meATP}$, and block by suramin ($\text{IC}_{50} \sim 1$ μM) and PPADS; these cells also express abundant P2X_2 receptor immunoreactivity, and it is concluded that homomeric P2X_2 receptors probably underlie the response (518). In more recent studies, Zhong et al. (517) have shown that guinea pig pelvic ganglion neurons differ substantially from those of the rat. Guinea pig cells exhibit responses consistent with homomeric P2X_3 , homomeric P2X_2 , and heteromeric $\text{P2X}_{2/3}$ receptors; individual cells can express more than one phenotype.

4. Enteric neurons

ATP evokes currents in guinea pig submucous neurons (11, 152) that reverse polarity at ~ 0 mV and are neither mimicked nor blocked by $\alpha\beta\text{meATP}$. In most (92%) neurons of the guinea pig myenteric plexus, ATP-evoked currents have many of the features of P2X_2 receptors, whereas the remaining 8% showed a quickly desensitizing current, mimicked by $\alpha\beta\text{meATP}$, and therefore similar to P2X_1 or P2X_3 receptors (519). The receptor on these cells is blocked by PPADS (10 μM) (12, 519), but reports differ regarding the effect of suramin (block, Ref. 144; potentiation, Refs. 10, 12). There are marked species differences in the sensitivity to suramin among P2X_4 receptors (145), and it would be interesting to determine the

suramin sensitivity of heterologously expressed guinea pig P2X₂ and P2X₄ receptors, to see if this might account for the phenotype of the native neurons. There are also slower responses to ATP in myenteric neurons, closing and opening of potassium channels, which presumably result from activation of P2Y receptors (10, 224).

A synaptic potential mediated by ATP has been described in guinea pig myenteric neurons (144, 519). The majority of fast excitatory synaptic potentials in myenteric plexus neurons are blocked by hexamethonium (10 μ M), but there remain some that are not blocked even by 300 μ M. In these cases, the resultant potential is blocked by suramin at concentrations similar to those required to block the depolarization evoked by exogenous ATP. LePard and Galligan (272) and Bian et al. (24) subsequently showed that ATP-mediated synapses are involved in the descending inhibitory pathway in the myenteric plexus. This provides the only clear example to date of ATP-mediated synaptic signaling between neurons in an identified physiological pathway.

5. Interactions with nicotinic receptors

Nakazawa et al. (325) first described how currents elicited by ATP in PC12 cells were not additive with those elicited by ACh. Although each receptor could be selectively blocked (by suramin and by hexamethonium), it was concluded that the "ATP-sensitive ionic pathway is not independent of the nicotine-sensitive pathway." The observations were later extended to sympathetic ganglion cells, where the interaction was shown to occur also in excised membrane patches (323, 327). It was concluded that the interaction might result from activation of one receptor leading to dephosphorylation of the other receptor, and hence a reduced current through it (324).

Essentially similar findings of current occlusion have been made for other sympathetic (guinea pig celiac ganglion, Ref. 410; see Ref. 411) and enteric (11, 152, 237, 520) neurons. Although there were some minor differences among the details in these reports, the main common findings were that the interaction seemed not to be at the level of the ligand binding, was not related to calcium entry, and did not require freely diffusible cytoplasmic messengers. On the other hand, the interaction was state dependent in that it required the receptors to be activated by their cognate ligands (237). The most likely interpretations are a direct protein-protein interaction between the channels or, as suggested by Nakazawa (324), an interaction in which the conformational change following ligand binding to one channel signals an alteration in the phosphorylation state of its neighbor. The physiological importance of such a direct postsynaptic interaction has not yet been addressed.

In summary, the most important results of functional studies on autonomic neurons are 1) the finding that

ATP-mediated synaptic transmission contributes to a defined neuronal pathway in the myenteric plexus, 2) the observations that individual neurons can express more than one form of P2X receptor which can be distinguished functionally, 3) the evidence that guinea pig and rat autonomic neurons assemble their P2X receptors from differing sets of subunits, and 4) the intriguing molecular interaction with nicotinic receptors that awaits a physiological interpretation.

F. Primary Sensory Neurons

1. Sensory fibers in the periphery

P2X receptors are expressed by subsets of primary afferent neurons (see Table 4 of review by Dunn et al., Ref. 104), and substantial evidence now implicates ATP in the initiation of impulses in some sensory fibers. Excitation of sensory neurons by ATP evokes a sensation of pain in humans (26, 176). In animals, afferent C fibers are directly excited by ATP and $\alpha\beta$ meATP (heart, Ref. 225; lung, Refs. 304, 365; esophagus, Ref. 354; joint, Ref. 102; intestine, Ref. 247; tongue, Ref. 388; skin, Ref. 174; bladder, Ref. 486; carotid body, Refs. 2, 515; vagus fibers, Ref. 208). In some of these cases, the effectiveness of $\alpha\beta$ meATP and the antagonism by TNP-ATP indicate involvement of a receptor that contains a P2X₃ subunit. The cell bodies of the peripheral fibers studied in these experiments are located in dorsal root ganglia or the nodose ganglion (e.g., heart, lung, esophagus, carotid body). Unfortunately, in most electrophysiological studies on the cell bodies, these have not been identified as belonging to any functionally or anatomically identified fibers in the periphery.

2. Cell bodies in nodose ganglia

Rat nodose ganglion neurons respond rather uniformly to ATP; the current shows little desensitization during applications of 1 s, and $\alpha\beta$ meATP is also a full agonist (233). This phenotype, a slowly desensitizing current evoked by either ATP or $\alpha\beta$ meATP, was the third main class of response observed at P2X receptors in native cells (445) and prompted the initial experiments that showed the formation of P2X_{2/3} heteromers (274). Thus the effectiveness of TNP-ATP as an antagonist closely parallels its action at heterologously expressed P2X_{2/3} heteromers, and the response to $\alpha\beta$ meATP is completely lost in nodose ganglion neurons from P2X₃ knockout mice (76, 433). On the other hand, individual nodose ganglion cells can express more than one P2X receptor. In many neurons, the current elicited by ATP is larger than that evoked by $\alpha\beta$ meATP, and experiments with TNP-ATP show biphasic inhibition curves that are well fit by a combination of P2X₂ homomeric and P2X₂/P2X₃ heteromeric channels (455) (Fig. 12).

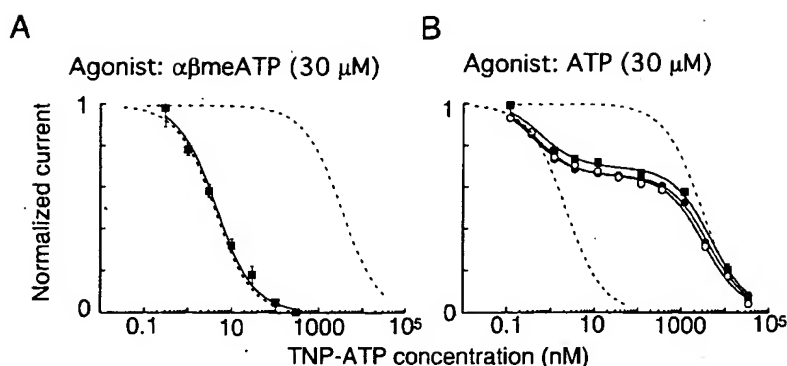


FIG. 12. Individual nodose ganglion neurons express more than one kind of P2X receptor. Graphs show the inhibition by TNP-ATP of currents recorded from nodose ganglion cells, elicited by $\alpha\beta$ -methylene ATP (A) or by ATP (B). Responses at homomeric P2X₃ receptors were desensitized by applying the agonists at 1-min intervals. A: when $\alpha\beta$ -methylene ATP is the agonist, the inhibition curve by 2',3'-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) is monophasic (consistent with a single class of P2X_{2/3} receptors). The IC₅₀ for TNP-ATP is ~3 nM. B: when ATP is the agonist, the inhibition curve by TNP-ATP is biphasic, indicating more than one class of receptor. The fits to the three individual cells shown indicate ~35% high affinity (IC₅₀ 3 nM) and 65% low affinity (IC₅₀ 3 μ M) forms. Broken lines indicate the inhibition curves for TNP-ATP at HEK293 cells transfected with P2X_{2/3} or P2X₂ receptors, taken from separate experiments. [From Thomas et al. (455).]

ATP-evoked currents in rat nodose ganglion cells are inhibited by magnesium (IC₅₀ ~1 mM) (278) and potentiated by zinc (up to ~5-fold; EC₅₀ ~10 μ M) (276), copper (280), and protons (277). Zinc and protons do not appear to act at the same site (277), which correlates well with recent work using mutagenesis on the cloned rat P2X₂ receptor (74) (see sect. 1B1).

3. Cell bodies in dorsal root ganglia

Dorsal root ganglion cells of the bullfrog were thoroughly studied by Bean et al. (17–19). The currents develop within 8 ms at saturating ATP concentrations (100 μ M), which was the limit of the solution exchange around an intact neuron. Careful concentration-response curves suggested that the binding of at least three molecules was required to open the channel. The currents exhibited strong inward rectification and in excised patches had an underlying unitary conductance of ~5 pS. Further pharmacological studies were carried out by Li, Weight, and colleagues (279, 281, 282). ATP elicits inward currents in acutely dissociated bullfrog cells (EC₅₀ ~5 μ M), which are mimicked by 2-MeSATP (EC₅₀ ~3 μ M), and $\alpha\beta$ meATP (EC₅₀ ~30 μ M) (281, 282), potentiated by protons (282),

and inhibited by zinc (IC₅₀ ~50 μ M, Ref. 281). This inhibition by zinc stands in contrast to the potentiation that is observed at mammalian P2X receptors, native (73) or cloned (511). The inhibition by zinc is prevented by treatment with dithiothreitol (281), suggesting that free sulfhydryl groups on the receptor may contribute to the zinc binding site; this is particularly interesting in view of the fact that all the P2X receptors have 10 conserved cysteines in their ectodomain.

Rat dorsal root ganglia were studied by Krishtal et al. (259) and by Jahr and Jessell (211), and these two reports provided the first evidence that ATP directly gates a cation-selective channel. More recent reports have attempted to define the subpopulation of neurons affected and to determine what might be the molecular composition of the P2X receptor (Table 6). Li et al. (279) used soma size and capsaicin sensitivity to classify acutely dissociated rat dorsal root ganglion cells. Small cells (<30 μ m diameter) were sensitive to capsaicin, and ATP (and $\alpha\beta$ meATP) evoked rapidly desensitizing currents [time constant of desensitization (τ_d) ~300 ms]. Medium-sized cells (30–50 μ m) were not affected by capsaicin; they showed a slowly desensitizing (τ_d ~1 s) current in re-

TABLE 6. Summary of effects of $\alpha\beta$ -methylene ATP on rat dorsal root ganglion cells, acutely dissociated

Class	$\alpha\beta$ -Methylene ATP	Kinetics	Capsaicin	Size	Isolectin B4
I	Insensitive		Insensitive	Large (>50 μ m)	Negative
II	Sensitive	Sustained	Insensitive	Medium (30–50 μ m)	Positive
III	Sensitive	Transient	Sensitive	Small (<30 μ m)	Positive

Proportions of the cells in the different classes differ among studies. Class II probably corresponds to neurons directly innervating lamina V cells; class III may correspond to neurons innervating more superficial lamina (see Ref. 322). For kinetics: transient, desensitization time constant <100 ms; sustained, desensitization time constant >1 s. (Data from Refs. 48, 160, 279, 469.)

sponse to ATP and $\alpha\beta\text{meATP}$. Large cells ($>50\ \mu\text{m}$) were unaffected by capsaicin or ATP. Ueno et al. (469) also used capsaicin sensitivity to classify rat dorsal root ganglion neurons; they described the population of capsaicin-insensitive cells that gave sustained responses to $\alpha\beta\text{meATP}$ ($\text{EC}_{50} \sim 60\ \mu\text{M}$) as well as a population of capsaicin-sensitive cells that gave rapidly desensitizing responses to $\alpha\beta\text{meATP}$ ($\text{EC}_{50} \sim 10\ \mu\text{M}$). Burgard et al. (49) recorded from cells which were stained with isolectin B4; this is a marker of a subset of sensory neurons generally thought to be involved in the sensation of acute pain (312), which is known to colocalize with P2X_3 receptor subunits (489). They also directly compared the responses with those observed in transfected cells. These cells exhibited fast- and slow-desensitizing responses; both were mimicked by $\alpha\beta\text{meATP}$ and blocked by TNP-ATP, and it was concluded that they correspond to P2X_3 homomeric and $\text{P2X}_2/\text{P2X}_3$ heteromeric channels, respectively. Responses to ATP with either of these kinetic phenotypes are blocked by nanomolar concentrations of ω -conotoxin GVIA, which is better known for its application to block N-type calcium channels (264).

Dorsal root ganglion cells removed from adults and maintained in tissue culture for 1–4 days have ATP-evoked currents that are much larger (several nanoamperes) than those observed in acutely dissociated ganglia (160); perhaps enzymatic treatment can inactivate the P2X receptors, which then need hours or days to reappear at the surface. Grubb and Evans (160) found that $>80\%$ of cells responded with a transient current, mimicked by $\alpha\beta\text{meATP}$ and blocked by TNP-ATP at subnanomolar concentrations, and thus resembling a homomeric P2X_3 receptor. However, many neurons also showed a component that desensitized more slowly, and which could be repeatedly evoked with repeated applications of ATP; this also suggests that single cells express more than one phenotypically distinct P2X receptor.

Neurons in adult dorsal root ganglia that have not been enzymatically dissociated and/or plated into short-term tissue culture rarely respond to ATP or $\alpha\beta\text{meATP}$ (437). It remains unclear whether the difference between intact ganglia and dissociated cells results from degradation of nucleotides in the intact situation, recording configuration (sharp electrode versus whole cell patch clamp), or stimulation of expression after plating onto glass coverslips. One explanation for the discrepancy might be that the intact ganglia are releasing sufficient ATP to desensitize the P2X receptors. For example, experiments on HL-60 cells showed that no responses to ATP could be elicited unless the cells were treated previously with apyrase, the interpretation being that the receptor could recover from desensitization if extracellular ATP was degraded (45). Other explanations might involve the influences of cell-cell interaction on P2X receptor subunit trafficking to the membrane.

There have not been systematic studies of the responses to ATP on dorsal root ganglion cells at different stages of development, but it is noted that results obtained on neonatal dorsal root ganglion cells sometimes differ from those observed in adults. Robertson et al. (385) and Rae et al. (375) used cells cultured from 1- to 6-day-old rats and found a response to ATP that closely resembled that seen at the homomeric P2X_3 receptor, including activation by $\beta\gamma\text{me-D-ATP}$ (although not $\beta\gamma\text{me-L-ATP}$) and Ap5A. Labrakakis et al. (261) found the two main classes of response to $\alpha\beta\text{meATP}$ (rapidly desensitizing and slowly desensitizing), as well as cells with mixed responses.

P2X_3 receptors are expressed immunohistochemically only by a subset of primary afferent neurons that has been implicated in nociception; these are mostly small-diameter cells that express receptors for isolectin B4 and capsaicin (TRPV1 vanilloid receptor), which do not contain the peptides substance P and somatostatin, which terminate in the inner part of lamina II, and which are dependent for survival on glial-derived neurotrophic factor rather than nerve growth factor (36, 60, 169, 489). Dorsal root ganglia from P2X_3 knock-out mice show no current in response to $\alpha\beta\text{meATP}$, consistent with the absence of any contribution of a P2X_3 subunit. There was a sustained response to ATP in the knock-out mice, indicating that other receptors (presumably containing P2X_2 subunits) functioned normally (76, 433). The absence of the P2X_3 receptor subunit from this subset of sensory nerves resulted in several phenotypic changes: 1) reduced nociceptive behavior to Formalin injection into the paw (76, 433), 2) reduced sensitivity to nonnoxious "warming" stimuli (433), 3) enhanced thermal hyperalgesia in chronic inflammation (76, 433), and 4) diminished reflex response to bladder distension (76, 486). The impairment of reflex bladder emptying confirms suggestions (127) that ATP released from the urothelium onto nearby primary afferent fibers is an initial stimulus leading from bladder filling to reflex autonomic emptying (486). It will be interesting to determine whether a similar ATP-dependent mechanism pertains in other hollow viscera such as gallbladder, intestine, and ureter.

The suggestion that ATP is released in conditions of inflammation has prompted examinations of its effects on the dorsal root ganglia that innervate inflamed tissues and interactions with the effects of other inflammatory mediators. Xu and Huang (512) showed that the responses of dorsal root ganglion cells removed from rats with inflamed paws were very similar to those from control rats, except that the currents were two to three times larger. There was also an upregulation of the amount of P2X_2 and P2X_3 proteins expressed by Western blotting. Substance P and bradykinin are potential inflammatory mediators; in oocyte expression, activation of these receptors can increase ATP-evoked currents at coexpressed P2X_3 and

P2X_{2/3} receptors (364), perhaps through receptor phosphorylation.

Further interactions have been reported between P2X receptors and other receptors on dorsal root ganglion cells. The more sustained responses to ATP of dorsal root ganglion cells, presumably mediated by the P2X_{2/3} heteromer, were decreased after treatment *in vitro* with a desensitizing concentration of capsaicin (369). This cross-desensitization was one way; responses to capsaicin were unaffected by prior treatment with ATP. It also required extracellular calcium and was blocked by intracellular BAPTA, leading to the conclusion that calcium entry through the activated capsaicin receptor led to the reduction in current through the P2X receptor. A related observation has been described by Sokolova et al. (425); they concluded that calcium entry through the activated ATP receptor inhibited currents at GABA_A receptors and that chloride efflux through GABA_A receptors inhibited currents at P2X receptors.

The increasing recent evidence for a role of ATP in initiating or enhancing inflammatory pain (175, 213, 467), taken together with the P2X₃ knock-out experiments (76, 433) and P2X₃ antisense oligonucleotide administration (192), strongly point to activation of the P2X_{2/3} heteromeric receptor being a critical early step in some aspects of pain sensation. Those expressed on capsaicin-sensitive C fibers include both rapidly desensitizing homomeric P2X₃ receptors and slowly desensitizing heteromeric P2X_{2/3} receptors; those expressed on capsaicin-insensitive Aδ fibers are heteromeric P2X_{2/3} receptors (Fig. 11).

4. Cell bodies in trigeminal ganglia

Cook et al. (84) showed that trigeminal ganglion neurons with projections from the tooth pulp, and therefore presumed to be functionally nociceptive, had responses to ATP and $\alpha\beta$ meATP. In some cells (28%), $\alpha\beta$ meATP elicited a rapidly desensitizing current, and in others (55%) the current was more sustained; this suggests that identified tooth pulp afferents may express channels as P2X₃ homomers or as P2X_{2/3} heteromers. In marked contrast, neurons with cell bodies in the mesencephalic nucleus of the Vth nerve (i.e., mechanosensitive Vth nerve primary afferent cells) responded to ATP but not $\alpha\beta$ meATP, indicating the absence of a P2X₃ subunit. Cells with the transient, rapidly desensitizing current showed a surprising effect of increasing the extracellular calcium concentration (from 1 to 10 mM) (83). This increased the amplitude of the current; other multivalent cations were also effective, particularly gadolinium which acted at 10 μ M. The effect of increasing the calcium concentration was remarkably long lasting; when the calcium concentration was raised to 10 mM for 2 min before (but not during) the ATP application, the effect of a subsequent ATP application was still enhanced. These

experiments suggest that there is a relatively high-affinity calcium (and gadolinium) binding site on the receptor ectodomain which, when occupied, enhances the ATP-induced current. The enhancement appears to result from an increased rate of recovery from desensitization.

5. Cell bodies in visual and auditory sensory ganglia

Ganglion cells cultured from rat retina responded to ATP, although amacrine cells did not (450). The inward current evoked by ATP (EC₅₀ ~10 μ M) was also evoked by ADP and $\alpha\beta$ meATP; it showed marked inward rectification and was carried by calcium as well as sodium ions ($P_{Ca}/P_{Cs} = 2.2$). The heterogeneity in terms of the effectiveness of $\alpha\beta$ meATP, and block by suramin, led the authors to conclude that more than one type of P2X receptor was expressed by the cells. The overall significance of these observations for retinal function is not yet clear, although it is known that ATP can be released from chick cholinergic amacrine-like cells (398). Primary afferent auditory neurons of the spiral ganglion exhibit inward currents typical of P2X receptors; these neurons are the cell bodies of auditory afferent nerves (397).

G. Epithelia and Endothelia

There is considerable evidence for autocrine/paracrine actions of ATP in epithelia, but for the most part these are thought to involve activation of P2Y receptors (liver, Ref. 403; pituitary, Ref. 61). There is, however, extensive evidence for the expression of functional P2X receptors on these tissues and in certain places (e.g., airway epithelia, kidney, vascular endothelium, ducted glands) key physiological roles are now being proposed.

1. Airway epithelium

Dissociated airway epithelial cells exhibit currents typical of P2X receptors. These are seen in freshly isolated tissue from rabbit airway (254, 297) as well as several other epithelial cell lines (453). In the case of the airways, considerable evidence implicates the P2X₇ subunit. The membrane currents are nondesensitizing and develop faster onset kinetics with repeated application (254), BzATP is more potent than ATP at causing a sustained increase in $[Ca^{2+}]_i$ (297), and extracellular sodium strongly inhibits the ATP response (293). Ciliary beat frequency increases as a result of calcium entry through the P2X receptor, and this effect is much enhanced at low extracellular sodium concentrations (293). The physiological implication is that locally released ATP, perhaps trapped by the mucus layer, acts back on P2X receptors to increase ciliary beat frequency.

2. Lacrimal gland

ATP activates 25-pS cation-selective channels in mouse (143, 399, 400) and rat (478) lacrimal acinar cells. A high concentration of ATP was required ($>300 \mu\text{M}$) to open the channels, but no further ATP analogs were tested that might help to identify the receptor involved. Procedures that activate protein kinase A within the cell, such as including the catalytic subunit of protein kinase A in the recording electrode, significantly potentiated the current. Because this effect was also seen in outside-out patches, it was considered that direct phosphorylation of the (P2X) channel by protein kinase A was the most likely interpretation (400). These experiments have been interpreted in the framework of corelease of ATP with norepinephrine from sympathetic nerves innervating the gland, in much the same way that ATP is coreleased with sympathetic nerves to certain smooth muscle effectors such as mesenteric arterioles and vas deferens.

3. Salivary glands

There has been substantial work on parotid acinar cells since the original observation by Gallacher (143) that ATP activates a rapid inward current. The channels are approximately equally permeable to sodium, potassium, and cesium and have $P_{\text{Ca}}/P_{\text{K}}$ of 2.3. Parotid acinar cells show an increase in $[\text{Ca}^{2+}]_i$ in response to ATP which depends on the presence of extracellular calcium (478); the pharmacological properties of the response were consistent with the involvement of P2X₄ and P2X₇ subunits (e.g., EC_{50} for BzATP was $3 \mu\text{M}$; Ref. 428), and these mRNAs but not others are expressed by the cells (454). The acini of submandibular glands isolated from rat exhibit responses to ATP and analogs that closely resemble those of cloned P2X₄ receptors, most notably the insensitivity to blockade by suramin (44). In the ductal cells, there is evidence for a P2X₇-like receptor that couples to kallikrein secretion through two phospholipase A₂ enzymes (5).

4. Exocrine pancreas

Duct cells of the exocrine pancreas express abundant P2X₄ and P2X₇ receptor mRNAs (182, 291), and luminal application of ATP and BzATP elicits a large depolarization with conductance increase (182). The limited concentration range of agonists used make it difficult to infer which subunits contribute to the receptor. On the same cells, activation of P2Y receptors by UTP caused a reduction in potassium conductance; on the basis of $[\text{Ca}^{2+}]_i$ measurements, P2Y receptors appear to be expressed on both luminal and basolateral membranes (291). Sorensen and Novak (429) have recently shown by direct measurement that ATP is released (by carbachol) from pancreatic acini and suggest that this may provide

the source of the ATP that reaches and activates P2X receptors on the duct cells (429). A resultant alteration in the properties of the duct cells might then have significant consequences for the composition of the pancreatic juice, but this is not fully understood.

5. Liver

Capiod (54) showed that ATP activates a cation-selective current in isolated guinea pig hepatocytes; the concomitant P2Y response that was otherwise present was blocked by intracellular EGTA. Low concentrations of ATP ($\sim 1 \mu\text{M}$) were effective, and $\alpha\beta\text{meATP}$ was about one-third as effective as ATP (at maximal $100 \mu\text{M}$ concentration) ATP. The conductance declined over a time course of several seconds. The current could also be carried by divalent cations, although the permeability of the substituting monovalent ion (NMDG) was not directly tested. The receptor is unusual in its high sensitivity to ATP, and the properties do not coincide with any of those yet studied by heterologous expression.

6. Anterior pituitary gland

An autocrine/paracrine role for ATP has been shown in the anterior pituitary. P2X₂ receptors are abundantly expressed in the pituitary gland (488), and this was the source used to clone the human P2X₂ receptor cDNA (292). P2X₇ receptors predominate on lactotrophs, whereas P2X₂ subunits are the only ones found on gonadotrophs and somatotrophs (see Ref. 439). In GH₃ cells, ATP (but not $\alpha\beta\text{meATP}$) elicits a nondesensitizing inward cation current, and this shows a progressive increase in permeability to NMDG; BzATP ($\text{EC}_{50} \sim 30 \mu\text{M}$) is considerably more effective than ATP ($\text{EC}_{50} \sim 1 \text{ mM}$), suggesting the involvement of a P2X₇ receptor (68).

7. Endocrine pancreas

The P2X₄ receptor cDNA was cloned from a rat pancreatic islet cDNA library, and insulin-secreting cell lines express P2X₄ receptors. Beta cells also release ATP, as detected with a nearby biosensor comprising P2X₂ receptors expressed on PC12 cells on a whole cell recording pipette held nearby (181). ATP depolarizes beta cells, increases $[\text{Ca}^{2+}]_i$, and promotes insulin release, but the receptors involved and other mechanistic aspects have not been worked out (283).

8. Renal epithelium

Schwiebert and Kishore (408) have recently reviewed the possible roles of P2X receptors in renal epithelium. Studies in renal epithelial cells lines (LLC-PK₁ cells, Ref. 136; mIMCD-K2 cells, Ref. 302) show the expression of several P2X receptors, and very high concentrations of ATP will induce apoptosis in rat cultured mesangial cells

(405). A cell line derived from mouse distal convoluted tubule cells expresses several P2X receptors (93). Application of ATP and some P2X-selective analogs inhibit magnesium uptake by the cells. A cohesive account of the functional role of P2X receptors in renal epithelium is awaited. As for the ducted glands, it will be important to test the hypothesis that ATP released into the lumen of the nephron has effects on luminal P2X receptors further along the nephron. The involvement of P2X receptors in paracrine signaling in the juxtaglomerular apparatus is presented elsewhere (see sect. *vH3*).

9. Sertoli cells

Extracellular ATP rapidly depolarizes Sertoli cells, increasing both $[Na^+]_i$ and $[Ca^{2+}]_i$ (140, 391), and this is consistent with the expression of P2X receptors in testis (290, 449). Isolated Sertoli cells secrete estradiol when stimulated with ATP; this requires extracellular sodium (although not calcium), suggesting that it more likely results from P2X rather than P2Y receptor activation.

10. Vascular endothelium

Ando and colleagues (514) have shown that exogenous ATP elicits an increase in $[Ca^{2+}]_i$ in vascular endothelial cells (514). They showed by RT-PCR that P2X₄ was by far the most abundantly expressed subunit in the cells. This expression could be reduced to ~25% of control by treatment with antisense oligonucleotides, and such treatment also much reduced the component of the increase in $[Ca^{2+}]_i$ that resulted from calcium entering the cell through P2X receptors. Because shear stress also causes an increase in $[Ca^{2+}]_i$, they hypothesized that this might result from an autocrine action of ATP. In support of this, they found that the shear stress-induced increase in $[Ca^{2+}]_i$ was also much inhibited by anti-P2X₄ oligonucleotides (513). The transcription of P2X₄ receptor genes (among others) is reduced by chronic shear stress, and this involves the transcription factor Sp1. This was shown by transfecting bovine endothelial cells with a construct containing the P2X₄ promoter (either wild type or with Sp1 binding site mutated) upstream of a luciferase reporter (253).

11. Retinal epithelium

The pigment epithelium of the rat retina also responds to ATP with an inward current and a rise in intracellular calcium (395); the current has many features of a P2X receptor (cation selectivity, rapid-onset kinetics), but the pharmacological characterization is not sufficient to make conclusions regarding the likely subtype.

12. Cochlea

The P2X₂ subunit and several of its splice variants were cloned from cochlea. There is evidence from recording membrane currents and/or imaging $[Ca^{2+}]_i$ for actions of ATP at P2X receptors on several cellular elements of the cochlea, including inner and outer hair cells (7, 320), cells of Reissner's membrane (separating the endolymph and perilymph, Ref. 246), Hensen's (262) and Deiter's (196) cells (which support the outer hair cells), stria vascularis (204), and spiral ganglion neurons (auditory primary afferent cells; see sect. *vF5*). In several of these studies, the P2X receptors have also been localized by immunohistochemistry, at the light and electron microscope level. The possible physiological roles for ATP in cochlear function have recently been reviewed (193, 194).

13. Skin

The skin of the larval bullfrogs responds to ATP applied to the apical surface. A sodium-dependent short-circuit current develops within a few hundred milliseconds and then desensitizes (90). The current occurs without change in intracellular calcium, and several features of the current are more typical of P2X rather than P2Y receptors (90, 91). A similar current has been reported for frog skin (42). A receptor cloned from tadpole (*Rana catesbeiana*) skin RNA is most similar in sequence to the P2X₅ family (214). When this cDNA was expressed in *Xenopus* oocytes, the currents had features of both P2X₅ and P2X₇ receptors, including propidium uptake. It is not really understood why tadpoles would respond to ATP; the suggestions of the authors range from detection of predators releasing ATP into the pond water to a role for locally released ATP trapped by a surface layer of mucus in the apoptotic death that occurs during metamorphosis.

It is interesting therefore that rat skin also expresses both P2X₅ and P2X₇ (but not other) receptor subunits (159), and human skin fibroblasts express P2X₇ receptors (426). In the case of the human fibroblasts, ATP and BzATP evoke depolarization (as measured with a potential-sensitive bisoxonol dye) as well as calcium and YO-PRO-1 uptake (401, 426).

H. Skeletomuscular Tissues

1. Bone

ATP stimulates bone resorption by osteoclasts (314). In rabbit osteoclasts, ATP and ATP γ S induce a cation current with many of the properties of heterologously expressed P2X₄ receptors, including rate of desensitization, potentiation by zinc, and insensitivity to suramin (318, 492). The inward current is followed by an outward potassium current, and this could be activated in isolation by adenosine 5'-O-(2-thiodiphosphate) (ADP β S) or UTP,

indicating the involvement of a P2Y receptor. A fragment of the rabbit P2X₄ receptor mRNA was amplified from the osteoclasts, and together with the pharmacological profile it appears that the ATP increases bone resorption by activating a receptor containing P2X₄ subunits. However, osteoclasts also express P2X₂ and P2X₇ subunits, as well as P2Y₁ and P2Y₂ receptors (34, 35). In the case of the P2Y receptors, the pharmacological profile (ADP is effective but UTP is not) suggests that activation of P2Y₁ receptors is responsible for stimulation of bone resorption (188, 189).

Some osteoblasts and osteoblast-like cells appear to express P2X₇ receptors. Application of BzATP to osteosarcoma cell lines (SaOS-2) and primary human bone-derived cells leads, in a subset of cells, to ethidium uptake, dramatic morphological changes, and eventual cell death (TUNEL staining and release of lactate dehydrogenase) (151). Osteoblasts also express several types of P2Y receptor (see Ref. 101).

2. Skeletal muscle

Some of the first evidence that ATP (1–10 μ M) directly gated ion channels was provided by recordings from 11-day-old chick embryonic skeletal myoblasts (43-pS single-channel conductance) and myotubes (48- and 30-pS conductances) (252). Similar effects were subsequently described for embryonic *Xenopus* muscle (60- and 41-pS conductances, Ref. 203). Thomas and Hume (456) recorded from myoballs cultured from 12-day-old chick embryos and reported that the ATP-activated channels were permeable to both cations and anions; the effect of ATP progressively disappears during embryonic life (from day 6 to day 17) but reappears in the adult after denervation (494). In adult rat muscle fibers, somewhat higher concentrations of ATP have been reported to increase the activity of nicotinic acetylcholine receptor channels (315). These observations take on particular interest in view of the abundance of P2X₅ and P2X₆ immunoreactivity in chick myoblasts (which disappears as myotubes form, Ref. 306), the cloning of the chick P2X₅ receptor from 10-day chick embryo skeletal muscle (28, 393, 394), and the recognition that the human P2X₆ receptor is heavily expressed in skeletal muscle (471). It is intriguing that the homomeric P2X₅ receptor is also significantly chloride permeable (394); comparison of the single-channel and pharmacological properties might indicate whether the native receptor comprises homomeric P2X₅ subunits or is a P2X_{5/6} heteromer.

3. Smooth muscle

A) VAS DEFERENS AND BLADDER. Although the P2X₁ receptor protein has a fairly widespread tissue distribution, it is best known for its high level of expression in smooth muscle tissue. This is because the vas deferens was the

original tissue for which ATP was proposed to be the main sympathetic transmitter (52, 50), and it was also the tissue for which this proposal was first substantiated electrophysiologically by the use of $\alpha\beta$ meATP as a desensitizing antagonist (424) and suramin (423) and PPADS (265, 303) as antagonists. Vas deferens or bladder removed from mice bred with a disrupted P2X₁ receptor gene show no contractions or inward currents when ATP is applied; they show no excitatory junction potential in response to stimulation of the sympathetic nerves to the vas deferens (316, 477). These mice have much reduced fertility, resulting from a reduced sperm count in the ejaculate, implying that the vigorous neurogenic contraction of the vas deferens plays a key role in normal ejaculatory function. These experiments indicate conclusively that the P2X₁ subunit is an essential component of the vas deferens P2X receptor. The results do not establish that the native receptor on smooth muscle cells is a homomeric P2X₁ form, as distinct from a heteromeric receptor containing one or more P2X₁ subunits. However, there are many similarities between the properties of the homomeric P2X₁ receptors in heterologous expression systems and those of the P2X responses observed in vas deferens smooth muscle. These include sensitivity to $\alpha\beta$ meATP, desensitization (236), single-channel properties (119, 331), and the effects of the somewhat selective antagonist Ip5I (197, 242).

Calcium ions contribute ~6% of the inward current evoked by ATP in bladder smooth muscle cells (404). The relative amounts of calcium entering through voltage-gated calcium channels and P2X receptors were compared by measuring $[Ca^{2+}]_i$. The calcium entry elicited by ATP (50 μ M, -60 mV) changed $[Ca^{2+}]_i$ from 130 to 730 nM, which was sufficient to inhibit profoundly the inward calcium current through the voltage-gated (L-type) calcium channels in the same cell.

B) VASCULAR SMOOTH MUSCLE. Arteries of the ear, tail, and mesentery have been extensively studied. The first reports that P2X receptors were permeable to calcium came from patch-clamp studies on the rabbit ear artery (21), and Ramme et al. (378) and Evans and Surprenant (123) provided conclusive evidence that ATP was the transmitter from sympathetic nerves to mesenteric arterioles. The receptor pharmacology seems very similar to the homomeric P2X₁ receptor (273, 275, 378, 524). TNP-ATP blocks the currents in dissociated mesenteric smooth muscle cells at a concentration of ~2 nM, and this is consistent with a P2X₁ receptor. However, in the intact tissue, the contraction elicited by $\alpha\beta$ meATP was blocked only by concentrations some 10,000 times higher. This difference might result from the TNP-ATP being broken down in the intact tissue (275), or it could be because the receptor activated by nerve-released ATP has a different subunit composition (and hence TNP-ATP sensitivity) than the

receptor activated by exogenous agonists applied to dissociated cells (see Ref. 447).

Human saphenous veins respond to ATP with an inward current and rise in $[Ca^{2+}]_i$ (56, 289). By RT-PCR they express mRNA for P2X₁ and P2X₇ subunits, but not P2X₃; other subtypes were not examined. The veins are contracted with either $\alpha\beta$ meATP or BzATP (10–100 μ M), but the inward currents elicited by the two agonists are quite distinct. $\alpha\beta$ meATP activated a rapidly ($\tau \sim 1.4$ s) desensitizing current, but BzATP evoked a current that desensitized little even in 3 min. BzATP was still effective in the sustained presence of $\alpha\beta$ meATP, indicating the activation of distinct sets of receptors. In parallel experiments, P2X₁ and P2X₇ subunits were coexpressed in COS cells, and the results were very similar: no currents were observed that could not be accounted for by the sum of those seen in COS cells expressing only P2X₁ subunits and COS cells expressing only P2X₇ subunits. The simplest interpretation of these results is that saphenous veins express independent (homomeric) P2X₁ and P2X₇ receptors.

P2X₁ receptors appear to be principally involved in the inward current and calcium entry in rat portal vein myocytes (310). The effect of ATP is mimicked by $\alpha\beta$ meATP (0.1–100 μ M) and, most convincingly, the inward current is not seen in cells recorded with pipettes containing an anti-P2X₁ subunit antibody. The calcium that enters the cells through P2X₁ receptors elicits further calcium release from intracellular stores. Confocal microscopy showed that these stores were distinct from those accessed by calcium entering through voltage-gated channels, and application of intracellular antibodies indicated involvement of ryanodine receptors type 2 but not type 3 receptors.

In the kidney, the smooth muscle cells of the preglomerular arterioles express P2X₁ receptors, but these are not seen on the postglomerular arterioles (58). When $\alpha\beta$ meATP is applied, these cells show a rise in $[Ca^{2+}]_i$ and contract; this requires extracellular calcium and is reversibly blocked by NF279 (207). Under normal conditions, most of the calcium that enters the cells appears to do so through voltage-gated L-type calcium channels activated by the P2X receptor-induced depolarization (497). It has been proposed that a paracrine action of ATP contributes to the tubuloglomerular feedback in renal vascular autoregulation (see Ref. 336). According to this hypothesis, ATP released from the macula densa results in the constriction of preglomerular afferent arterioles.

C) GASTROINTESTINAL SMOOTH MUSCLE. The toad stomach has been studied by Singer and colleagues (470, 525). ATP activates a nondesensitizing, nonselective cation current in isolated cells, and this has many of the pharmacological characteristics of a P2Z or P2X₇ receptor. In the same cells, this cation current was followed by a potassium current; this had the same pharmacological properties

with respect to the ATP, but it was not wholly due to calcium entering through the P2X channel. The potassium channels involved were identified as fatty acid activated channels, suggesting that activation of the P2X₇ receptor resulted in the generation of a lipid second messenger.

4. Cardiac muscle

The actions of ATP on the heart have recently been reviewed by Vassort (474) and are therefore presented very briefly here. ATP activates a cation conductance in isolated myocytes from frog atrium (139); rat (67), rabbit, and guinea pig (187, 360) ventricle; and rabbit sinoatrial node (415). Many of the properties of the currents described are consistent with the involvement of a P2X receptor, and the ineffectiveness of $\alpha\beta$ meATP (139, 360, 415) might point to P2X₂- or P2X₄-containing subtypes. There is immunohistochemical evidence for several P2X receptors in rat cardiac myocytes (177, 488), and mRNA for P2X₁, P2X₂, P2X₄, and P2X₅ receptors can be found in rat (146, 345, 431) or human heart (96).

ATP increases the force of contraction of isolated cardiac muscle fibres (371) and of intact heart (305). This effect is mimicked by 2-MeSATP but not $\alpha\beta$ meATP and insensitive to block by suramin (371), consistent with involvement of a P2X₄ receptor. Similar results have recently been reported for chick myocytes, with the additional observation that the effects of ATP were lost in cells treated with oligonucleotides antisense to the chick P2X₄ receptor RNA (198). It is now important to determine whether calcium entry through the P2X₄ receptor is essential for the increased force of contraction, as well as uncovering the source of the extracellular ATP under more physiological circumstances.

I. Hemopoietic Tissue

1. Mast cells

ATP degranulates and releases histamine from mast cells, and it stimulates the labeling of phosphatidylinositol; these effects require extracellular calcium and occur within minutes (77–79). ATP also causes leakage from the cells of intracellular nucleotides and phosphorylated metabolites; this action occurs only with longer exposure to ATP and does not require calcium. Cockcroft and Gomperts (79) studied the actions of ATP in a range of calcium and magnesium concentrations and concluded that all three actions resulted from activation of the same receptor and that ATP⁴⁻ was probably the active ligand. In these experiments, as in all subsequent studies of this kind, the interpretation that the active ligand is ATP⁴⁻ rests on the assumption that the only effect of altering the concentrations of extracellular magnesium and calcium is to change the concentrations of the various forms of ATP.

Cell permeabilization by ATP was further characterized by Bennett et al. (22) and Tatham et al. (451), who used it to load mast cells with molecules up to 600–1,000 Da in molecular mass. Gordon (154) suggested that this receptor be termed the P2Z receptor. The only detailed electrophysiological study was by Tatham and Lindau (452). They showed that ATP evoked an inward current that developed with the time course of the solution exchange (~100 ms). In the absence of divalent cations, the EC_{50} for ATP was ~20 μ M (i.e., the ATP^{4-} concentration). There was little decline in the current during applications of several minutes. The maximal conductance increase evoked by ATP was very large, up to 50 nS, and the current-voltage plot was close to linear. Experiments in which the extracellular concentration of both sodium and chloride were reduced to one-fifth showed that the permeability increase involved both cations and anions ("weak cation selectivity"), but the possible permeability to larger organic cations was not directly examined.

Osipchuk and Cahalan (350) showed that ATP released from one mast cell could diffuse several tens of micrometers to elicit rises in $[Ca^{2+}]_i$ in surrounding cells. However, as for similar paracrine signaling reported in the liver (403) and among glial cells (85, 170), this seems to involve P2Y rather than P2X receptors.

2. Macrophages and related cells

Several measures of the action of extracellular ATP have been applied to macrophages and related cells (e.g., the mouse cell line J774, human monocytes and monocyte-derived macrophages, the human monocyte cell line THP-1, microglia, mouse microglia NTW cells, human macrophage cell line U937, mouse macrophage cell line BAC1.2F5, and human monocyte-derived dendritic cells). These include membrane current (43, 47, 63, 87, 118, 171, 227, 268, 332–335, 344, 380, 446, 484), increase in $[Ca^{2+}]_i$ (23, 88, 132, 157, 202, 402, 406), uptake of fluorescent dyes (65, 168, 184, 185, 202, 402, 427, 438, 444, 446), membrane blebbing or other morphological change (80), spontaneous cell fusion (65, 125), interleukin processing and release (43, 129, 130, 155, 158, 366), activation of NF- κ B (131, 133), killing of *Mycobacterium tuberculosis* (260, 267, 422), activation of p38 MAP kinase (186), activation of phospholipase D (111, 112, 200), formation of multinucleate giant cells (65, 125), and various measures of cell death (see Ref. 100).

Evidence for the involvement of the P2X₇ receptors in these effects is substantial. This usually takes the form of 1) effective concentrations of ATP are in the hundreds of micromolar, 2) BzATP is 10- to 100-fold more effective than ATP, and 3) the responses to ATP and BzATP are much increased by reducing the concentration of extracellular divalent cations. Further evidence comes from the use of antagonists. The most commonly used are

oxidized ATP (although this is not selective for P2X₇ receptor; see Ref. 348) and KN-62; blockade of responses by a monoclonal antibody has also been reported (43). The most useful antagonist now available, at least for rat P2X₇ receptors, is Brilliant Blue G (215). The most definitive way to show P2X₇ receptor involvement, in the mouse, is to demonstrate the loss of the effect in a P2X₇ receptor-deficient mouse; this has been shown for IL-1 β secretion (427). None of these approaches demonstrates that the macrophage receptor is a homomeric P2X₇ receptor but, because P2X₇ subunits did not interact with other P2X subunits in a biochemical assay (462), it is often assumed that this is the case.

A) MEMBRANE CURRENTS. There are many similarities between the properties of the whole cell current observed in J774 cells and in heterologously expressed P2X receptors when ATP or BzATP is applied; in addition to those mentioned above, these include cation selectivity, lack of rectification, little or no desensitization over tens of seconds, and progressive increase in permeability to NMDG (446). On the other hand, rat peritoneal macrophages were found to be impermeable to Tris, at least with a low ATP concentration (3.5 μ M applied for 10 s) (332); concentrations above 500 μ M were reported to "permeabilize" the cells, but no details of this are provided (332). A conductance that has properties very similar to that activated by ATP can also be activated by including guanosine 5'-O-(3-thiotriphosphate) (GTP γ S) in the recording pipette (333, 334). Coutinho-Silva et al. (86) used mouse peritoneal macrophages and described the activation by ATP of a 7.8-pS channel that did not discriminate among cations. A similar channel in thymic reticulum macrophages had a conductance of 5 pS.

Coutinho-Silva et al. (87) made cell-attached recordings from mouse peritoneal macrophages and J774 cells. They were able to activate single channels in the membrane patch by applying ATP to the rest of the cell (i.e., away from the patch-clamped membrane). This action of ATP had all the hallmarks of P2Z or P2X₇ receptor involvement. These results imply that an intracellular second messenger liberated by P2X₇ receptor activation is able to activate channels under the patch-clamp electrode. The unitary currents recorded were very large, with linear current-voltage relations corresponding to conductances of ~400 pS. Unitary currents of broadly similar properties were seen with Tris or NMDG as the main cation, or glutamate as the main anion, in the pipette. The currents required several seconds to activate and activated much more quickly at higher temperatures (30–37°C). Unfortunately, in such experiments when activity is recorded in the cell-attached configuration, it is difficult to conclude that the second messenger is liberated by a process specific to P2X receptors, rather than simply by the membrane depolarization or calcium entry that follows P2X receptor activation.

Microglia from the brain of neonatal mice (171) and rats (484) clearly show two discrete currents in response to ATP. At concentrations lower than 100 μM , ATP activates an inward current that 1) reverses at ~ 0 mV and shows inward rectification, 2) desensitizes during several seconds, and 3) is not blocked by oxidized ATP (300 μM). At a concentration of 3 mM, ATP activates a current that 1) reverses at ~ 0 mV but shows no rectification, 2) does not desensitize during tens of seconds, and 3) is 90% blocked by oxidized ATP (484). These observations strongly suggest that the cells express two sets of P2X receptors; the first has properties similar to homomeric P2X₂ or P2X₄ receptors, and the second resembles homomeric P2X₇ receptors. A microglia-derived cell line (NTW8) exhibits currents with several pharmacological features of P2X₇ receptors (63). The kinetics and amplitude of the currents change with repeated application; in low concentrations of divalent ions, the currents elicited by ATP increase in amplitude with repeated applications (63). However, human monocyte-derived macrophages cultured for 5–7 days appear to show mostly the P2X₂/P2X₄-like current component (118).

B) UPTAKE OF CALCIUM AND FLUORESCENT DYES. There are several reports of calcium (or barium) entry elicited by ATP and analogs, and these generally have the features expected of P2X₇ receptor activation (157, 126, 132, 311, 406). Uptake of ethidium and YO-PRO-1 has also been extensively studied (47, 65, 168, 184, 185, 202, 402, 427, 438, 444, 446). There is the potential to obtain mechanistic information from this type of experiment, by measuring the detailed kinetics of uptake and using fluorescent probes with a range of molecular sizes. This has not been much exploited.

Nuttall and Dubyak (349) originally provided evidence that the ionic current activated by ATP in macrophages (the channel) was different in its properties from the dye-entry pathway (the pore). Recently, Dubyak and colleagues (401, 402) have shown that the “channel” (i.e., calcium entry) and the “pore” (i.e., ethidium uptake) in THP1 monocytes and fibroblasts can be distinguished in several ways. First, maitotoxin activates both pathways, as does BzATP at P2X₇ receptors, but the actions of maitotoxin do not involve the P2X₇ receptor. Like BzATP, maitotoxin exposure eventually leads to cell death (release of lactic dehydrogenase). This work strongly supports the hypothesis that maitotoxin acting through its own yet-unidentified receptor, and ATP acting through the P2X₇ receptor, both result in the activation of a common pore. If the pore corresponds to the 400-pS channel of Coutinho-Silva et al. (87), this suggests the involvement of diffusible cytoplasmic messenger. Several candidate messengers are suggested by the recent identification of proteins in HEK293 cells that interact with the P2X₇ receptor. These could include phosphatidylinositol 4,5-bisphosphate generated by the action of phosphatidylin-

sitol-4-kinase, with subsequent activation of phospholipase D (239). This attractive hypothesis can only be substantiated by the identification of the pore molecule itself, as well as the transduction mechanism from the P2X₇ receptor.

C) OTHER DOWNSTREAM SIGNALS. We have discussed the ionic current (channel) and the dye uptake (pore). Macrophages and related cells also undergo cytoskeletal rearrangements and release interleukins when activated by ATP. In THP1 cells, the former is evidenced by the appearance of large membrane blebs (1 to >10 μm). The identification of α -actinin and β -actin among the proteins that associate with the P2X₇ receptor suggests a route to membrane blebbing (see sect. IVK2) (239). The IL-1 β release occurs in lipopolysaccharide-primed cells. It has recently been shown that this occurs by the shedding of microvesicles (<1 μm diameter) from the cell surface (294). These are shed within 10–30 s of applying BzATP, as evidenced by 1) a reduction in membrane capacitance and 2) the release of labeled lipid particles into the medium. Even within 10 s of applying BzATP, the THP1 cells “flip” their phosphatidylserine to the outer leaflet of the membrane, where it becomes accessible to labeling with rhodamine-annexin. The released vesicles also have exposed phosphatidylserine and can be collected on annexin-coated beads. Lysis of the vesicles showed them to contain IL-1 β , and this was shown to be bioactive by adding vesicles to HeLa cells expressing the IL-1 receptor coupled to a luciferase reporter assay (294). Convincing evidence that the P2X₇ receptor is required for the release of IL-1 β from lipopolysaccharide-primed macrophages has been provided by the complete absence of any effect of ATP in macrophages from P2X₇ receptor knock-out mice (427).

3. Lymphocytes

Peripheral blood lymphocytes and lymphocytes from patients with chronic lymphatic leukemia (CLL) have been extensively studied by Wiley et al. (503). Immunohistochemical studies suggest that they express P2X₁, P2X₂, P2X₄, and P2X₇ subunits (419). The expression of P2X₇ receptors by B lymphocytes is about one-third that observed for peripheral blood monocytes, similar to that of NK lymphocytes, and somewhat greater than that of T lymphocytes. They have the experimental advantage that they show no P2Y responses. The sequelae of activating P2X₇ receptors on lymphocytes include 1) increase in $[\text{Ca}^{2+}]_i$ or $[\text{Ba}^{2+}]_i$ by entry from the extracellular solution (163, 504–507), 2) uptake of ethidium or YO-PRO-1 (163, 504, 505), 3) activation of phospholipase D (128, 149), 4) shedding of L-selectin and CD23 (212), and 5) stimulation of mitogenesis (14). Each of these effects shows the hallmarks of P2X₇ receptor involvement; BzATP is more

potent than ATP, and responses are potentiated by magnesium removal.

The divalent ion entry is inhibited by extracellular sodium (506) and by KN-62 ($IC_{50} \sim 20$ nM) (150) as well as by receptor blockers such as oxidized ATP (506). The ethidium uptake begins some 30 s after the entry of divalent cations, and the delay is longer with lower agonist concentrations or lower temperatures; it is also potentiated by reducing the extracellular sodium concentration and blocked by KN-62 (150, 505, 508). In these respects the properties of human lymphocytes mirror closely those of HEK cells (202, 307, 380) and *Xenopus* oocytes (229, 363) expressing P2X₇ receptors (but see Refs. 248, 470).

The activation of phospholipase D and the shedding of L-selectin are also inhibited by extracellular sodium ions and blocked by KN-62 ($IC_{50} \sim 10$ nM) (149, 150, 165). Gargett et al. (149) indicate that phospholipase D activation results from the entry of calcium through the P2Z receptor, but this is in clear contrast to the findings in a mouse macrophage cell line (111). Leukocytes that have shed L-selectin will adhere less well at inflammatory sites (153), and it will be important to work out the molecular mechanisms that couple the activated P2X₇ receptor to L-selectin shedding. One contribution to the loss of L-selectin might be the microvesicle shedding recently described for THP1 cells and transfected HEK cells (294).

Tonsillar B cells as well as human B lymphocytes immortalized by Epstein-Barr virus have been studied by patch-clamp recordings (41, 298, 299). In both cases, BzATP ($EC_{50} \sim 15$ μ M) or ATP ($EC_{50} \sim 100$ μ M) elicited opening of a 9-pS channel that was permeable to small cations, including calcium, but not to choline. The whole cell currents showed little rectification and no desensitization during recordings of several minutes. These cells show no evidence of developing an increased permeability to larger organic cations, and application of ATP and analogs did not lead to the release of intracellular fluo 3.

Thymocytes include T cells at various stages of maturation ($cd4^-cd8^-$ to $cd4^+cd8^+$). All classes of cells respond to extracellular ATP with an increase in $[Ca^{2+}]_i$ (69), with double positive cells the least responsive. This $[Ca^{2+}]_i$ signal results from entry of external calcium rather than release from stores (390) and was more pronounced in the larger, actively dividing thymocytes compared with smaller terminally differentiated cells (390). Freedman et al. (137) patch-clamped mouse thymocytes (double positive or double negative) and showed that $\alpha\beta$ meATP evoked a small rapidly desensitizing current, whereas ATP⁴⁻ (i.e., ATP in magnesium-free solution) elicited a sustained nonselective cation current (and $[Ca^{2+}]_i$ signal). This suggests the expression of P2X₁ and P2X₇ receptors, and RT-PCR indicated the presence of mRNA for P2X₁, P2X₂, P2X₆, and P2X₇ subunits. Because PPADS blocked the effects of ATP, they tested the effect

of more continuous exposure to PPADS on thymocyte development. This supported an earlier study in which high concentrations of P2X receptor antagonists protected thymocytes from cell death (70). Taken together with the fact that the P2X₁ receptor cDNA (partial) was first isolated from thymocytes induced to undergo apoptosis (353), and the observation that extracellular ATP can promote thymocyte death (319, 370), the studies suggest a possible role for extracellular ATP and P2X receptors in T-cell selection and maturation (but see Ref. 218). Thymocytes do not exhibit any ethidium influx when challenged with BzATP (202, 370).

In T lymphocytes from peripheral blood, extracellular ATP stimulates mitogenesis, and the antagonist-oxidized ATP decreases proliferation (14). This suggested an autocrine role for released ATP in the control of cell growth. In support of this view, the proliferation in serum-free medium of a lymphoid cell line not normally expressing P2X₇ receptors can be sustained by transfection with the receptor. Oxidized ATP again has its antiproliferative action in such transfected cells, but not in untransfected controls (15).

In conclusion, the inward current evoked by ATP in macrophages and their progenitors, and in lymphocytes, can result from activation of P2X receptors that may or may not contain the P2X₇ subunit. In those cases where the evidence for P2X₇ receptor involvement is the strong, the application of ATP may or may not lead to cell "permeabilization"; the increase in permeability to large cations (NMDG; and fluorescent dyes such as ethidium and YO-PRO-1) is seen in some (mast cells, monocytes, macrophages, peripheral blood lymphocytes) but not other (T cells, tonsillar B cells) native cells. The most likely explanation for this is that other molecules are required in addition to the P2X receptor to form the dye-permeable pathway; these may interact with the P2X receptor and allow it to increase in diameter, or they may be independent pore molecules activated by an intracellular signaling pathway initiated from the P2X₇ receptor (Fig. 10). The physiological role of this increased permeability to large molecules remains as mysterious as it was when first described by Cockcroft and Gompert (78) more than 20 years ago. Several further downstream signaling events have been described; it remains to be shown whether these are in any way caused by the initial inward current, or the permeabilization, or whether they represent additional somewhat independent consequences of liganding the receptor (see Ref. 239).

4. Platelets

Platelets express P2X₁ subunits (443), and their electrophysiological response to nucleotides closely resembles that of homomeric P2X₁ receptors (295). ADP has been known classically as the purine that elicits platelet

aggregation, and this is generally believed to involve P2Y₁ and P2Y₁₂ receptors (see Ref. 16). Controversy persists as to whether ADP can activate platelet P2X receptors. A recent paper on the characterization of the platelet receptor drew attention to the dangers of using impure commercial preparations of nucleotides (296), showing that actions ascribed to ADP were not observed after it was purified. On the other hand, Greco et al. (156) found a splice variant of the P2X₁ receptor to be abundant in human platelets. This variant lacks 17 amino acids at the beginning of exon 6, and the difference appears to have a large effect on the agonist selectivity of the receptor. When the mutant form was expressed in 1321N1 astrocytoma cells, ADP and ATP, but not $\alpha\beta$ meATP, were effective to evoke calcium influx. These authors suggest that this mutant form may contribute to the ADP-sensitive calcium entry pathway, either as a homomer or a heteromer with wild-type subunits; electrophysiological studies would be helpful in this regard.

A clever method of measuring the concentration of ATP was introduced by Dubyak and colleagues (20). They made a chimeric protein from the IgG binding domain of protein A and firefly luciferase, which then attached specifically to cells treated with antibody to a given cell surface protein. They coated platelets with anti-CD41 antibody, and thus measured the ATP concentration in the vicinity of the plasma membrane. After treatment with thrombin, this rose from undetectable to $\sim 16 \mu\text{M}$, well within the range that would activate P2X₁ receptors.

VI. PERSPECTIVE

Important advances in understanding have accrued on several fronts since the cloning of cDNAs in 1994. Heterologous expression and mutagenesis have identified parts of the subunits likely to contribute to key functions, such as subunit multimerization, ATP binding, channel gating, and ion permeation. On the other hand, simple questions remain unresolved. How can the replacement of an -O- atom in ATP by -CH₂- have such profound consequences for receptor agonism in some but not other P2X receptors? What molecular structure underlies the potent effects on the receptors of certain extracellular ions? How is the permeation pathway formed? The next horizon in this direction must be structural studies on parts or all of the receptor protein.

The failure to discern any relationship to other known families of ion channels is a major handicap in our understanding of the more fundamental biological aspects of P2X receptors, as is the apparent restriction of the channel family to vertebrates, given that many experimental approaches to the molecular physiology, including structural studies, would be facilitated by simpler animal models. Nucleotide signaling by cAMP

is well known in amoebae, but this involves a seven-transmembrane receptor; emerging invertebrate genomes must be searched for P2X receptor relatives.

The identification of posttranslational modifications is beginning to indicate how channel function can be modified by other cellular components. Conversely, activation of the P2X₇ receptors not only opens a channel but engages several downstream effectors. Although the cloning of the P2X₇ subunit cDNA provided a cation-permeable channel with distinctive properties, it has not provided a full explanation of cell "permeabilization" by extracellular nucleotides, or of the coupling of P2Z receptors to these other cellular effectors. The isolation of the first members of a signaling complex of proteins that interact with this receptor promises to reveal how other molecular players are influenced by the P2X₇ receptor.

The study of the P2X receptors continues to be hampered by the lack of potent and selective antagonists. Studies on cloned receptors have allowed some progress to be made here, and this promises to accelerate as more high-throughput screens are run in the search for potential antagonists that might provide the starting point for new therapeutics.

Antibodies derived on the basis of deduced amino acid sequences have revealed an unexpectedly wide tissue distribution of P2X receptors. However, more and better antibodies are needed to address the cell biology of the receptors. How are they trafficked and assembled in cells? Might P2X receptors play key signaling roles in intracellular organelles? As for most other multimeric ion channels, a key issue remains knowledge of the subunit composition of native receptor(s) in individual cells.

The physiological role of P2X receptors on native cells is becoming clearer through the effects of agonists and antagonists and the defects observed following block of gene expression. The peripheral nervous system leads the way. ATP operates as a synaptic transmitter from sympathetic nerves to some smooth muscle, and in a descending inhibitory pathway in the gut wall. A role for the P2X₃ subunit is clear in the sensation of some forms of inflammatory pain and mechanical allodynia, and compelling evidence exists for other mechanosensing functions in autonomic viscera such as the bladder. Although several effects (presynaptic, postsynaptic) of ATP can be observed on central neurons, nowhere in the central nervous system is there a clearly understood picture of the physiological significance.

ATP is increasingly realized to be an autocrine and paracrine transmitter, and P2X receptors seem likely to be involved here in ducted glands, airway epithelia, and perhaps the kidney. Finally, considerable progress has been made in understanding some of the roles of ATP in immune cells and inflamed tissues, and particularly the way in which P2X₇ receptors elicit the release of cytokines.

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